Acrylamide in food products: Identification, formation and analytical methodology

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Abstract

The aim of this thesis was to verify the indicated occurrence of acrylamide formation in heating of food, to identify factors affecting the formation, and to identify important sources of acrylamide exposure from food.

As a prerequisite for the studies, gas- and liquid-chromatographic methods with mass spectrometric detection were developed for the analysis of acrylamide in food. The developed methods showed a high correlation coefficient (0.99), high sensitivity and reproducibility.

Acrylamide was demonstrated to occur in heated food products, with unexpectedly high levels in potato products (up to mg/kg level in potato crisps) and in beetroot. The identity of acrylamide was confirmed by these developed methods.

With potato as a food model, different factors affecting the acrylamide formation were tested. It was shown that the addition of asparagine and fructose, as well as heating temperature and time had a large impact on the formation. Other factors affecting the acrylamide content were pH, addition of other amino acids apart from asparagine, protein and other reducing sugars. No significant effects were observed from addition of neither antioxidant nor radical initiators.

It was discovered that acrylamide could be formed during heating of biological materials similar to food, also at temperatures below 100 ˚C. Furthermore, it was demonstrated that a fraction of acrylamide evaporates during heating, similar to conditions for cooking in household kitchens, and during dry matter determinations in laboratories (65-130 ˚C). This constitutes an earlier unobserved source of exposure to acrylamide.

The method for extraction of food was studied with regard to yield of acrylamide. It was shown that the yield at pH ≥12 increases 3 - 4 times compared to normal water extraction for some foods products. Extraction at acidic pH or with enzymatic treatment was also tested, showing no effect on yield.

In a study with mice the bioaviability of acrylamide extracted with the normal water extration and at alkaline pH was compared. It was shown that the extra acrylamide released at alkaline pH gave insignificant contributions to the *in vivo* dose, measured by hemoglobin adducts.

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Table of content

List of original papers

The present thesis is based on the original papers below, which are referred to in the text by their Roman numerals.

Paper I Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. & Törnqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *Journal of Agricultural and Food Chemistry,* **50**, 4998-5006.

Paper II Rydberg, P., Eriksson, S., Tareke, E., Karlsson, P., Ehrenberg, L. & Törnqvist, M. (2003). Investigations of factors that influence the acrylamide content of heated foodstuffs. *Journal of Agricultural and Food Chemistry,* **51**, 7012-7018.

Paper III Eriksson, S., Karlsson, P. & Törnqvist, M. (2005). Measurement of evaporated acrylamide during heat treatment of food and other biological materials. *LWT – Food Science and Technology,* submitted.

Paper IV Eriksson, S. & Karlsson, P. (2005). Alternative extraction techniques for analysis of acrylamide in food: Influence of pH and digestive enzymes. *LWT – Food Science and Technology*, in press/on-line 15 April 2005.

Paper V Vikström, A., Eriksson, S., Paulsson, B., Karlsson, P. & Törnqvist, M. (2005). Comparison of bioavailability from different foods – A study in mice. *Xenobiotica*, submitted.

Abbreviations

APCI	Atmospheric pressure chemical ionization
ASE	Accelerated Solvent Extraction
a_w	Activity of water
BMAA	β-*N*-methylamino-L-alanine
BRC	British Retail Consortium
BSTFA	*N,O*-bis (trimethylsilyl)trifluoroacetamide
BTMSA	*N,O*-bis(trimethylsilyl)acrylamide
CIAA	Confederation of the food and drink industries of the EU
DON	Deoxynivalenol
DSP	Diarrheic shellfish poisoning
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAPAS	Food analysis performance assessment scheme
GC	Gas chromatography
GC-EC	Gas chromatography with electron capture detector
HACCP	Hazard analysis and critical control point
Hb	Hemoglobin
HCA	Heterocyclic amines
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HSPME	Headspace solid-phase microextraction
HT-2	HT-2 toxin
IARC	International Agency for Research on Cancer
INFOSAN	International Food Safety Authorities Network
JECFA	Joint FAO/WHO Expert Committee on Food Additives

JRC	European Commission's Directorate General Joint Research Centre
LAL	Lysinoalanine
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MEEKC	Microemulsion electrokinetic chromatography
3-MCPD	3-Monochloropropane-1,2-diol
MOE	Margin of exposure
MS	Mass spectrometry
N/A	Not applicable
NFA	National Food Administration, Sweden
NOEL	No observed effect level
OEL	Occupational exposure limit
PAH	Polyaromatic hydrocarbons
PCB	Polychlorinated biphenyls
PFOA	Perfluorooctanic acid
PFOS	Perfluorooctane sulfonate
PSP	Paralytic shellfish poisoning
SPE	Solid phase extraction
SU	Stockholm University
SWEDAC	Swedish Board for Accreditation and Conformity Assessment
T-2	T-2 toxin

1 Introduction to the subject

The analysing of foods has gone through a lot of changes during the last decade. In the past it was concerned mainly with the analysis of known compounds, such as nutrients in nutrient declarations, and other product specifications. In western countries, where nutrients and vitamins are nearly unlimited, there has been a change towards discussing the safety of foods, and the analysis of unknown risk factors in a product, i.e. to investigate products from a consumer risk point of view. This change has taken place because of many alarms, consumers perception about risks, media alerts, frauds, new analytical possibilities and the debate concerning healthy and unhealthy foods.

In the food sector society around the world, the risk concept is behind the need for quality and traceability systems like Hazard Analysis and Critical Control Point (HACCP), and similar regulations like British Retail Consortium (BRC), Global Standards (BRC Global Standard Food; BRC/IoP Global Standard Food Packaging), and the new ISO 22000:2005 (Food safety management systems – Requirements for any organization in the food chain). The risk concept is also the driving force behind the EU food regulation EU Regulation 882/2004 (EU Commission, 2004) and the formation of European Food Safety Authority (EFSA) in 2002.

1.1 Chemical Risk Factors in Food

Risk factors in food are either from chemical or microbiological origin, or a combination of both. Some of the major groups of chemical risk factors, except toxins from bacteria, are listed below.

1.1.1 Naturally occurring compounds from toxin-producing organisms

Toxins from moulds: Currently a few hundred mycotoxins are known, which are often produced by the genera *Aspergillus*, *Penicillium* and *Fusarium*. The most prominent toxins are aflatoxins, deoxynivalenol (DON), zearealenone, ochratoxin, fumonisin and patulin. Analytical methods for many of these compounds have been of interest for a long time, and many types of analytical methodologies are available (Frisvad & Thrane, 1987; Lauren & Agnew, 1991; van der Gaag, *et al*., 2003), and the most toxic and common are included in the EU Directive 466/2001 (EU Commission, 2001).

Mushrooms toxins (Faulstich, 2005): The largest number of fatalities due to ingestion of mushrooms is caused by amatoxins and in a majority of cases by the death cap (*Amanita phalloides,* Sw. lömsk flugsvamp). Also for the white species, the destroying angel (*Amanita virosa,* Sw. vit flugsvamp) fatal cases are

reported. The toxin orellanine is exclusively found in the mushrooms of genus *Cortinarius*, including (*Cortinarius speciocissimus,* Sw. toppig giftspindelskivling). Toxicity mainly develops in the kidney, leading to renal failure. The toxin gyrometrin, a formyl-methylhydrazone of acetaldehyde, is produced by the false morel (*Gyromitra esculenta,* Sw. stenmurkla). In the cooking process as well as in the gastrointestinal tract, gyrometrin is hydrolysed into formyl-methylhydrazine, and further to mono-methylhydrazine, which represents the real poison. In severe cases a hepato-renal phase could lead to liver injuries.

Marine phycotoxins in seafood (Backer, *et al*., 2005): The paralytic shellfish poisons (PSP), include at least 20 derivatives of saxitoxin, which is a tetrahydropurine comprising two guanidinium functions. Saxitoxins are produced by dinoflagellate species from *Alexandrium*, *Pyrodinium* and *Gymnodinium* genera. Paralytic shellfish poisons accumulate in many higher organisms that eat these microalgae. Saxitoxin has a relaxant action on vascular smooth muscle.

The toxin group diarrhetic shellfish poisons (DSP) are produced primarily by dinoflagellates from the genera *Dinophysis*. The toxins include a series of polyether molecules (including okadaic acid and six derivatives of dinophysistoxin), four pectenotoxins (polyether lactones), and yessotoxins (including two sulphate esters that resemble brevetoxins). Okadaic acid directly stimulates smooth muscle contraction and probably causes diarrhea.

β-*N*-methylamino-L-alanine (BMAA): This compound is a neurotoxic nonprotein amino acid. It occurs both free and could be released from a bounded form by acid hydrolysis. BMAA may be produced by all known groups of cyanobacteria, including cyanobacterial symbionts and free-living cyanobacteria with increasing concentrations in the food chain (Cox, Banack, & Murch, 2003; Murch, Cox, & Banak, 2004; Cox, *et al*., 2005).

1.1.2 Other toxic compounds accumulated in food under non-optimal production or storage

Heavy metals: Lead, arsenic and cadmium etc. could become concentrated in food products, when food is produced in an un-efficient way (Schrenk, 2004). Higher uptake has been observed in children for lead and cadmium, especially in industrial areas, and in humans, who require high food intake, compared to FAO/WHO recommendations (Protasowicki, 2005).

Biogenic amines: When food products or raw materials for food production are stored in non-optimal ways, compounds like biogenic amines could be formed (Flick, Jr. & Granata, 2005). Exposure leads to symptoms such as gastrointestinal, circulatory, or cutaneous with individual patterns of

susceptibility. Antihistamines may be used effectively to treat the symptoms. In addition, the biogenic amines cadaverine, putrescine and histamine may be produced post-mortem from non-protein bounded lysine, ornithine and histidine.

1.1.3 By human added risk factors

There are general pollutants, which are not directly added into the food, but can be found in food as contaminants. Those types of compounds include polyaromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and dioxins. Another type is contaminants from package material used for food products, which include phthalates, perfluorooctane sulfonate (PFOS), perfluorooctanic acid (PFOA), semicarbazide and others (Schrenk, 2004). By human introduced risk factors into the food chain also involve antibiotics, pesticides and other chemical groups.

1.1.4 Compounds formed during processing of food products

A fourth group includes components that are formed during the processing of food products, either in an industrial scale or at home. This includes 3-monochloropropane-1,2-diol (3-MCPD) that may be formed in a wide variety of industrial and domestically produced foods and food ingredients. They were first reported in acid-hydrolyzed vegetable proteins (Velisek, *et al*., 1978).

Heating of food can also produce toxicants like PAH, which are known to be produced in grilling through pyrolysis and pyrosynthesis. At high temperatures, such as conditions for incomplete combustion (400-1000 ºC), organic compounds easily can fragment into smaller compounds, mostly radicals, which may then recombine to form a number of relatively stable PAHs (Jägerstad & Skog, 2005).

In addition, a range of compounds is formed in the temperature-dependent Maillard reaction. Some of these are listed below.

Heterocyclic amines (HCA): Four classes of them occur in heated/cooked meat: pyrido-imidazoles/-indoles, quinolines, quinoxalines and pyridines (Skog, Johansson & Jägerstad, 1998). They are formed through pyrolyzed amino acids such as tryptophan, glutamic acid, lysine, phenylalanine, creatinine and ornithine. Adding small amounts of certain carbohydrates may be a simple and effective way of reducing the amount of HCA in households and commercial preparations of beef burgers (Persson, *et al*., 2004).

Furan is the parent compound of the class of derivates known as furans. Furan is considered “possible carcinogenic to humans” by IARC (Hoenicke, *et al*., 2004a; Ho, Yoo & Tefera, 2005). The formation through Maillard reactions has shown to be possible from ascorbic acid, glycolaldehyde/alanine, erythrose,

ribose/serine, sucrose/serine, fructose/serine and glucose/cysteine (Perez Locas & Yaylayan, 2004).

Hydroxymethylfurfural (HMF) is formed during the advanced step in the Maillard reactions, and can be used as a useful indicator for control of the cooking processes in cereal products (Ait Ameur, *et al*., 2004). HMF is reported to be slightly mutagenic, but its toxicological relevance is still not clarified (Janzowski, *et al*., 2000).

Lysinoalanine (LAL) is formed in a two-step process: First, formation of a dehydroalanine intermediate. Second, reaction of the double bound of dehydroalanine with the ε-NH_2 group of lysine to form lysinoalanine. LAL is reported to induce enlargement of nuclei of rats and mice kidney cells but not in primates (Friedman, 1999).

1.1.5 Acrylamide

One of the latest discovered neurotoxic and carcinogenic substances in food is acrylamide. My thesis will concentrate on acrylamide in food products, its identification, formation and methods for the analysis.

2 Introduction to the thesis

Acrylamide became an issue to the Swedish people and as well as for the Dept. of Environmental Chemistry, Stockholm University (SU) and AnalyCen Nordic AB in 1997. Because of a large water leakage during the building of a railway tunnel through Hallandsås, a mountain ridge in the south west of Sweden, a grouting material had to be used to seal the tunnel walls. This grouting agent (Rhoca Gil; Rhone-Poulenc), containing monomeric acrylamide and *N*-methylolacrylamide, was used at a quantity of 1400 tones during August and September 1997. In September an acute situation arose, with the observation of dead fish and paralysed cattle near the construction site (Tunnelkommissionen, 1998). A large leak of un-polymerized acrylamide and *N*-methylolacrylamide into the environment appeared to be the cause, and the acrylamides spread into streams, ground water and wells, causing concern about exposure to residents in the area and also to the tunnel workers. Through measurement of reaction products (adducts) with the protein haemoglobin (Hb) in blood at the Dept. of Environmental Chemistry, SU, it was shown that many of the tunnel workers had received high exposures and several of them developed peripheral nerve symptoms similar to those reported from acrylamide poisoning (Hagmar, *et al.,* 2001). As a consequence of the environmental contamination and of the consumer resistance to food products from the area, cattle were taken away and local food products were destroyed (Tunnelkommissionen, 1998; Boija, 1998). However, to my knowledge, acrylamide was never discovered in food products produced in the contaminated area. The construction of the tunnel was abandoned, and was not started again until 2004.

AnalyCen, Lidköping, were performing analysis of water and of solid materials like soil, sediment and food products from the Hallandsås area. The laboratory became accredited for analysis of acrylamide and *N*-methylolacrylamide in water down to 0.5 µg/L, which at that time was sufficient (SWEDAC, 1998). The methodology used for solid material was a modification of the method for analysis of acrylamide in water. Figure 2.1.

For the characterization of the exposure situation, acrylamide was measured *in vivo* as adducts to Hb at the Dept. of Environmental Chemistry (Hagmar, *et al*., 2001; Godin, *et al*., 2002). The method had earlier been applied to studies of occupational exposure to acrylamide (Bergmark, *et al*., 1993; Bergmark, 1997). These measurements of Hb-adducts in blood samples from cattle, free-living animals and humans from the Hallandsås area, also gave a focus on a probable

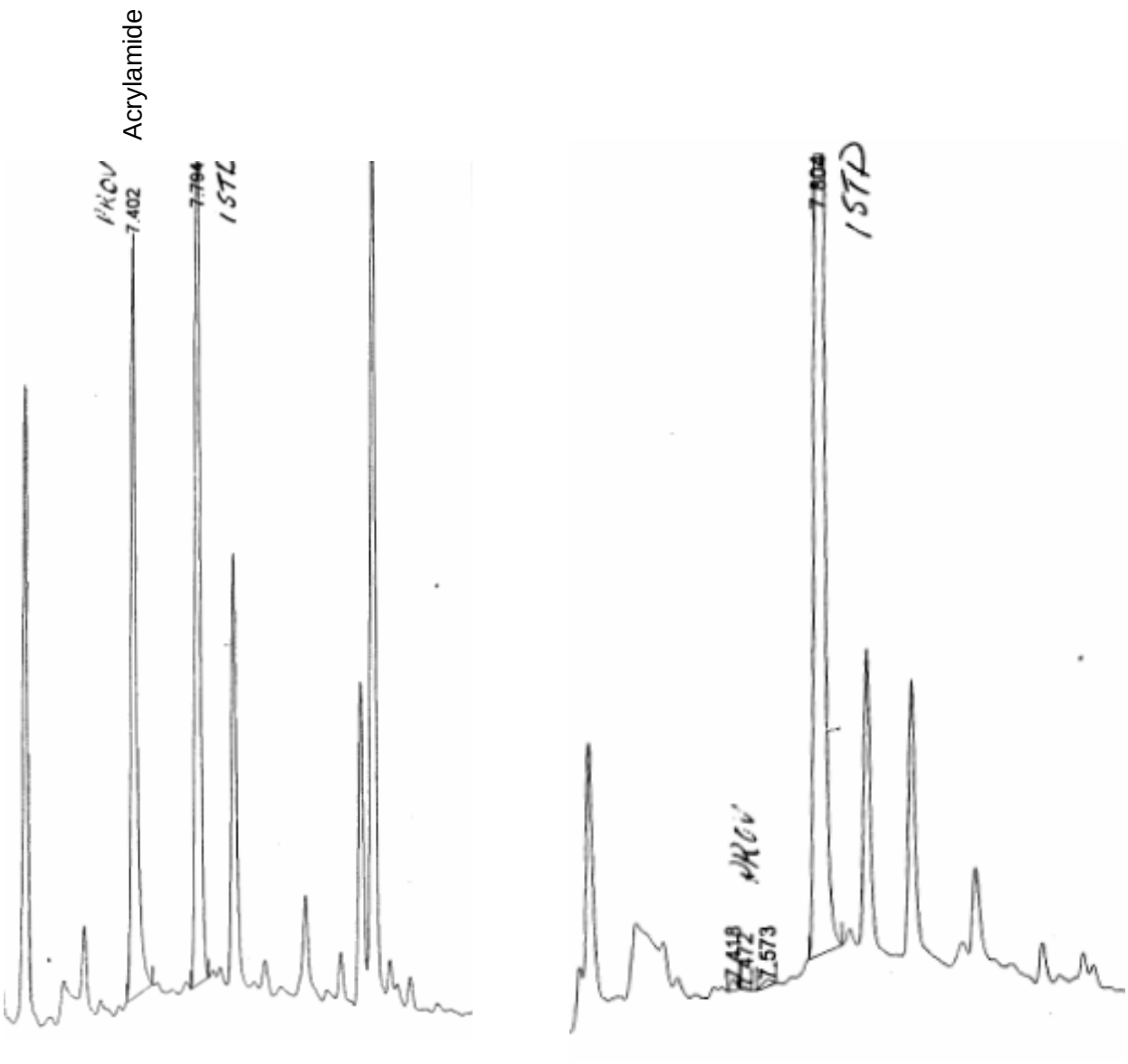

Figure 2.1 Acrylamide in water from Hallandsås with GC-EC after bromination. Left one containing acrylamide (ret. time 7.40 min), and right one without acrylamide.

general background exposure to acrylamide in humans (Törnqvist, 2005). This resulted in tests of heated food as a possible source of acrylamide exposure carried out in animal experiments at Stockholm University (Tareke, *et al*., 2000). In this study AnalyCen contributed with analysis of acrylamide in the heated animal feed. The method used for acrylamide analysis in feed was a modification of the method used for analysis of samples from the Hallandsås event and published in this paper (Tareke, *et al*., 2000).

The results in the animal feeding study strongly indicated that acrylamide could be formed in heating of food and indeed we also published a preliminary result on acrylamide content in hamburgers as a footnote in this paper (Tareke, *et al*., 2000). The story about acrylamide in heated food started with these experiments in 1998. According to preliminary estimates (Bergmark, 1997; Törnqvist, *et al*.,

1998), the background Hb-adduct level of acrylamide in humans could correspond to a relatively high uptake of acrylamide (about 80-100 µg/day). This was of great concern since acrylamide is classified as ”probably carcinogenic to humans” by International Agency for Research on Cancer (IARC) (IARC, 1994). This initiated the active search for the reason for the acrylamide background.

The remarkably high levels of acrylamide in certain food products was observed for the first time by AnalyCen and Dept. of Environmental Chemistry on 9 January 2001, when we found unexpectedly high levels of acrylamide (around 700 µg/kg) in heated mashed fried potato (Figure 2.2). These results are included in **Paper I** in this thesis.

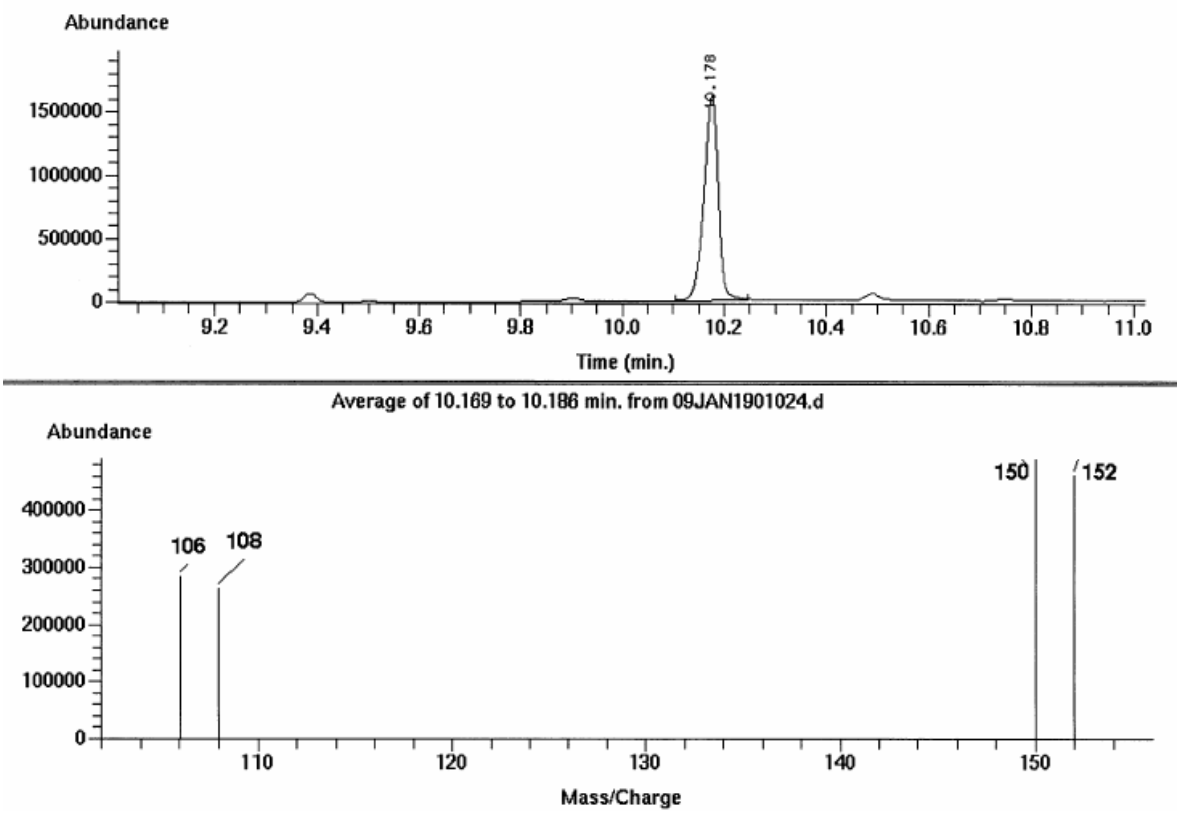

Figure 2.2 First evidence of high acrylamide content in food. Acrylamide at 10.178 min (about 700 µg/kg) in upper chromatogram, determinated as 2,3-dibromopropionamideby GC-MS.

My thesis discusses our results and puts it in relation to the unusually large quantity of knowledge concerning acrylamide in food, data that has been obtained through intensive research activities around the world, during the relatively short period of time since our initial discovery and during the work with this thesis. The excessive amount of new findings in this field also obviously interacted and gave input to our own research.

3 Objectives

The overall objectives of my thesis was to:

- Verify the formation of heat-induced acrylamide in food.
- Characterize determinants for the formation.
- Characterize and quantify content of acrylamide in foods.
- Evaluate exposure to acrylamide from food preparation.

Specific aims of each paper:

Paper I

Develop analytical methodologies for the analysis of acrylamide in food.

Verify possible occurrence of acrylamide in food.

Quantify the levels of acrylamide in different types of foods.

Paper I, II

Identify determinants for the formation of acrylamide in food.

Paper III

Clarify whether formed acrylamide in food could evaporate during heating and contribute to exposure.

Paper IV

Clarify whether the extraction methods in the analysis influences measured level of acrylamide in food.

Paper V

Clarify whether the acrylamide measured at extraction at high pH is bioavailable.

4 Acrylamide background

Moureu produced for the first time technic acrylamide in Germany 1893. In 1952, Hercules Company started making research quantities of acrylamide, and in 1954, production for commercial use started. At that time, this was the only known acrylamide exposure from industrial products, since the use of acrylamide was primarily for the production of polyacrylamides with widely different physical and chemical properties (Smith & Oehme, 1991).

4.1 Chemical characteristics of acrylamide

Acrylamide (CH_2=CH-CO-NH_2; 2-propenamide) is a white crystalline solid with a molecular weight of 71.08. It has a melting point of 84.5±0.3 °C, low vapour pressure (e.g. 0.007 mm Hg at 25 °C, 0.03 mm Hg at 40 °C, 0.07 mm Hg at 50 °C and 0.14 at 55 °C), a high boiling point (136 °C at 3.3 kPa/25 mmHg) (Norris, 1967; American Cyanamid, 1969; Habermann, 1991). The solubility in polar and unpolar solvents varies considerably, and the solubility in water is extremely high, see Table 4.1. Many factors have effect on the strategi to use for the analytical methods: the solubility, the low molecular weight and the low volatility.

Table 4.1 Solubility of acrylamide in different solvents (Habermann, 1991; American Cyanamid, 1969).

Solvents	**g/100 mL at 30º C**
Water	215.5
Methanol	155
Dimethyl sulphoxide	124
Dimethyl formamide	119
Ethanol	86.2
Acetone	63.1
Pyridine	61.9
Acetonitrile	39.6
Ethylene glycol monobuthyl ether	31
Dioxane	30
Ethyl acetate	12.6
Chloroform	2.66
1,2-Dichloroethane	1.50
Benzene	0.35
Carbon tetrachloride	0.038
n-Heptane	0.0068

Acrylamide is a difunctional monomer, containing a reactive electrophilic double bond and an amide group. The limited conjugation involving an π-electrons means that acrylamide lacks a strong chromophore for UV detection and does not fluoresce. Acrylamide exhibits both weakly acidic and basic properties. The electron withdrawing carboxamide group activates the double bond, which reacts with nucleophilic regents by 1,4-addition reaction mechanisms. Many of these reactions are reversible, and the rate of reaction depends on the strength of the nucleophile. Examples are the addition of ammonia, amines, phosphines and bisulphites. Alkaline conditions permit the addition of mercaptans, sulfides, ketones, nitroalkanes, and alcohols to acrylamide (Habermann, 1991).

4.2 Toxicity

Acrylamide is a compound, with a potential to cause a spectrum of toxic effects (IARC, 1994; European Union Risk Assessment Report, 2002; Manson, *et al*., 2005), including neurotoxic effects as has been observed in humans. Acrylamide has also been classified as a "probable human carcinogen" (IARC, 1994). The mutagenic and carcinogenic properties of acrylamide are assumed to depend on the epoxy metabolite, glycidamide (reviewed by Rice, 2005).

4.3 Occurrence

The wide-spread polymers of acrylamide have had a range of applications in water and waste-water treatment, crude oil production processes, paper and paper pulp processing, mineral processing, concrete processing, as cosmetic additives, in soil and sand treatment, coating application, textile processing and other miscellaneous uses (photographic emulsion, adhesives and coatings) (Smith & Oehme, 1991). Formation in tobacco smoke has been known, but not seen as a major problem in tobacco smoking (Schumacher, *et al.,* 1977; deBethizy, *et al*., 1990; White, *et al*., 1990; Smith, *et al*., 2000). It can be analyzed either in the smoke or by Hb adduct measurements in smokers (Bergmark, 1997; Schettgen, *et al*., 2003). Analysis of snuff and tobacco can be analyzed with the methods as used for food products, Chapter 6.

4.3.1 Contaminations to the environment

In the past, it has been contaminations of the acrylamide monomers in the environment through use of polyacrylamides in china clay and paper industry, and by water industry as polymer flocculants (Brown, Rhead & Bancroft, 1980; Bachmann, Myers & Bezuidenhout, 1992). Later grouting operations, when polyacrylamide has been used as the grouting material, have led to leakage of monomers, which has contaminated the environment, as well as affected workers (Mapp, *et al*., 1977; Cummins, *et al*., 1992; Weideborg, *et al*., 2001;

Hagmar, *et al*., 2001; Kjuus, *et al*., 2004). Also, during similar circumstances when contaminations of water have occurred, exposed humans have shown symptoms of poisoning (Igisu, *et al*., 1975).

4.4 Analytical techniques for acrylamide in water and polyacrylamide

Even though there have been microbiological methods for analysis of acrylamide in waste water (Ignatov, *et al*., 1996), the detection limit (10 mg/L) has not been sufficiently low to make these methods useful for environmental samples. Analysis of acrylamide in water has been performed for a long time, with different types of techniques, including both gas chromatography (GC) and liquid chromatography (LC) methodologies. Extraction of acrylamide in technical products, i.e. polyacrylamide products, needs a different approach, and cannot be performed in the same way as for water or food products.

4.4.1 Gas chromatography (GC)

Acrylamide can be converted to 2,3-dibromopropionamide, by bromination of its double bound, see Chapter 6. This bromination was in the past made by irradiation with ultraviolet light (Croll & Simkins, 1972), but later changed to an ionic reaction (Hashimoto, 1976; Arikawa & Shiga, 1980), which is still the most common reaction. 2,3-Dibromopropionamide is volatile and can be detectable on a GC with an electron capture detector or an alkali flame-detector (Tekel, *et al*., 1989; U.S. EPA, 1996). The use of packed columns was replaced with capillary columns, and it was suggested to perform the analysis on the more stable 2-bromopropenamide, obtained after debromination of 2,3-dibromopropionamide (Andrawes, Greenhouse & Draney, 1987; Martin, Samec & Vogel, 1990). Applications of mass spectrometry (MS)-detection (Prezioso, *et al*., 2002) increased the possibility to analyze drinking water according to EU regulation for drinking water 98/83/EC (EU Commission, 1998). The legal limit is 0.1 μg/L, which refers to the residual acrylamide monomer concentration in the water as calculated according to specifications of the maximum release from the corresponding polymer in contact with water. The sensitivity of the methodology has been further improved by use of negative chemical ionization (Morizane, Hara & Shiode, 2002), use of SPE columns for concentration of samples (Kawata, *et al*., 2001) or by utilizing a more sensitive derivatization technique performing the analysis on a tandem mass spectrometry instrumentation (Licea Pérez & Osterman-Golkar, 2003).

4.4.2 Liquid chromatography (LC)

Acrylamide does not show any specific wavelength absorption maxima, i.e. measurement at a universal wavelength (general for most kinds of compounds, normally below 220 nm) is possible for measurement. Even though the

sensitivity has not been sufficient in most cases, UV-detection has been applicable for degradation studies of acrylamide in soils and for measurements at other occasions, when higher concentrations of acrylamide were expected in the environment (Skelly & Husser, 1978; Lande, Bosch & Howard, 1979; Shanker, Ramakrishna & Seth, 1990; Smith & Oehme, 1993; Ver Vers, 1999; Saroja, Gowda & Tharanthan, 2000; U.S. EPA, 1994). By performing the same type of bromination as for GC analysis, there was possible to achieve a detection limit down to 4 µg/L, still by using low UV-detection (Brown & Rhead, 1979; Brown, *et al*., 1982). Today, a detection limit of 0.5 µg/L has been achieved by direct injection on a LC-MS of the brominated derivate (Cavalli, Polesello & Saccani, 2004), which is not far away from the EU 98/83 drinking water directive. Thermospray interface connected to a LC-MS, was reported to have a detection limit as low as 0.2 µg/kg, when analysing acrylamide in sugar (Cutié & Kallos, 1986).

4.4.3 Analysis of monomeric acrylamide in polyacrylamide

Contaminations of acrylamide monomers in polyacrylamide led to development of different methods for acrylamide analysis in of polyacrylamide matrices. These methods are not optimized for analysis of other types of products, since the main problem is to extract the free acrylamide out of the polyacrylamide (MacWilliams *et al*. 1965; Betso & McLean, 1976; Skelly & Husser, 1978; Tseng, 1990; Castle, 1993; Smith & Oehme, 1993; Hernández-Barajas & Hunkeler, 1996; Ver Vers, 1999; Saroja *et al*. 2000). Acrylamide monomer in polyacrylamide can also be analyzed without performing extraction by use of pyrolysis-solvent trapping-gas chromatography (Wang & Gerhardt, 1996).

Polyacrylamide is used in packing material for food products. The monomer acrylamide is in this occasion classified as an indirect food additive, when it migrates into the food from the polyacrylamide containing packing material. The leakage of acrylamide to food products has a limit of 10 µg/kg (EU Commission, 2002a), analyzed in food and/or food simulators. Methods for extraction are standardized (EN 1186-1, 2002; EN 13130-1, 2004), and analytical instrumental methodology is also in the standard as a technical specification (CEN/TS 13130-10, 2005). My opinion is that this technical specification, most likely will be neglected, compared to the new methods normally used for analysis of acrylamide in foods, which has been developed during the last years (Chapter 6).

Polyacrylamide has been used in the cosmetic industry for a long time (Isacoff, 1973). Today, polyacrylamide hydrogel is used in ophthalmic operations, production of contact lenses, ingredient of microencapsulated gelospheres for drug treatment, filler in the cosmetic industry, used in plastic and aesthetic surgery like breast augmentation in some countries (Cheng, *et al*., 2002;

Christensen, *et al*., 2003; Patrick, 2004). In cosmetic products, it is a maximum permitted residual content of 0.1 mg/kg for body-care leave-on products and 0.5 mg/kg for other products (EU Commission, 2002). In the US, the mean usage of cosmetic products per day for women has been estimated to about 10 g (Loretz, *et al*., 2005).

Analytical methods for acrylamide in water and a few GC methods for food products were published before our findings of heat-induced formation of acrylamide in foods, Chapter 5, **Paper I**. The findings of acrylamide in food had to go in parallel with development of analytical methods, which could confirm the appearance of acrylamide in foods, Chapter 6, **Paper I**.

5 Acrylamide in foods (Paper I)

On the basis of Hb-adduct measurements in humans, cattle and free-living animals, as summarized in Chapter 2, a general exposure source to humans of acrylamide was indicated. The adduct level in humans was estimated to correspond to a relatively high background exposure. With regard to the cancer risk estimates of acrylamide, it was of importance to trace the source of exposure. Several observations led to the hypothesis that heating of food could be an important source (Törnqvist, 2005). There were strong indications from the initial animal experiments with fried feed and the preliminary analysis on fried hamburgers, that acrylamide could be formed during heating of food (Tareke, *et al*., 2000). These initial findings called for verifications, and broader investigations.

5.1 Levels

To investigate whether acrylamide could be formed in foods, a range of cooking experiments and analytical work was performed in cooperation between Dept. Environmental Chemistry, SU, and AnalyCen Nordic AB, Lidköping. These cooking experiments were done under controlled laboratory conditions at SU and the analysis of formed acrylamide in various food products were performed at AnalyCen.

We discovered that acrylamide was formed in different food types, independently, if the heating/frying was done with a frying pan, in an oven, or by microwave heating. Unexpectedly high levels of acrylamide were found in potato products. In raw or boiled food products, no acrylamide was detected, **Paper I**.

Potato products and other heated foodstuff products were obtained from restaurants or from grocery stores. Samples were analyzed for acrylamide levels and compared with laboratory-prepared foodstuffs. High acrylamide contents were also found in the selected commercial foods. Details of the pre-purchase processing were not available, and variations with respect to composition and cooking etc. method were not considered. Also, in the evaluation of analytical methods, commercial foodstuffs were used for analysis, since analytical methods were developed/validated in parallel as the experiments were performed, Chapter 6. Table 5.1 summarizes results from **Paper I**.

Table 5.1 Measured levels of acrylamide in foods presented in **Paper I.**

Type of food (Number of samples/analysis)	**Acrylamide content; median (range)** (µg/kg heated food)
Laboratory-fried protein-rich food	
Beef, minced (5)	17 (15 – 22)
Chicken, minced (2)	28 (16 – 41)
Cod, minced (3)	< 5 (< 5 – 11)
Laboratory-fried carbohydrate-rich food	
Potato, grated (5)	447 (310 – 780)
Beetroot, grated (2)	850 (810 – 890)
Boiled or raw food	
Potato, beef, cod (12)	< 5
Restaurant-prepared etc. foods	
Hamburger (4)	18 (14 – 23)
French fries (6)	424 (314 – 732)
Potato crisps (8)	1740 (1300 – 3900)
Crisp bread (different types) (3)	208 (37 – 1730)
Beer (3)	< 5

Since then, thousands of samples have been analyzed for acrylamide at AnalyCen, with the in Chapter 6 described methods.

There are now innumerable numbers of results reported on acrylamide in food from all over the world, either in scientific publications or on the web (Lineback, *et al*., 2005).

5.2 Identification

The findings of unexpected (and unbelievable) high levels of acrylamide in some basic food products (up to mg/kg levels), led to the awareness that very strong evidence would be required to prove the occurrence and identity of acrylamide in food products. Confirmation of the results by two completely different analytical methods would be necessary. We used two methods based on MS techniques combined with purification with solid phase extraction (SPE). The findings were supported by the quantification of acrylamide in various foodstuff matrices by using the two different developed procedures for purification and instrumental chromatography (GC/LC), with different detection principles (MS or MS/MS), with and without bromination **(Paper I)**.

- GC-MS, where the analysis involved bromination at low pH and analysis of brominated samples at high temperatures. In the procedure for GC-MS analysis acrylamide was derivatized to 2,3-dibromopropionamide by bromination of the ethylenic double bond (Chapter 6.2.1).
- Analysis by LC-MS/MS involves direct determination of underivatized acrylamide. The LC-MS/MS method was more lenient with respect to direct measurement without prior derivatization and no increase in temperature during the chromatographic separation.
- In both methods ($^{13}C_3$)acrylamide was used as an internal standard and measured as brominated derivative (GC-MS) or without derivatization (LC-MS/MS), respectively.
- Analysis by an LC-MS/MS method (monitoring of product ions of a precursor ion, MRM) used for the underivatized acrylamide in this study gave a stronger evidence of the identity than the GC-MS analysis on brominated derivate only. The acrylamide content in potato products was verified by recording product ion spectra of respective analyte in LC-MS/MS, and mass spectrum in GC-MS analysis on the brominated product. The spectra are identical for the standards and the analytes. The results were comparable between the methods with and without derivatization. This was a strong support for the identity of the analyte. In addition, a further support for the identity was that several ions were monitored for the analyte in both the GC-MS analysis and the LC-MS/MS analysis and that the relative ion abundances are the same for the analyte as for the standard. Further explanation of the ions is presented in Chapter 6.
- The analysis of acrylamide had been performed using four different GC columns and two different LC columns (Table 5.2); columns that also were used later by other laboratories performing acrylamide analysis (see

Chapter 6). Independent of the utilized separation methods, the analytes exhibited the same retention times as the corresponding internal and external standards.

- The levels of acrylamide in food obtained with the methods were in agreement. The LC-MS/MS values were found to be 0.99 [0.95-1.04; 95% confidence interval] of the GC-MS values
- The identification of acrylamide in food products was confirmed!

Table 5.2. Retention times obtained when analysing 2,3-dibromopropionamide and acrylamide with different columns by the GC-MS and the LC-MS/MS method, respectively. Stated retention times are approximate values. In all cases the analyte co-eluted with the chosen internal standard, **Paper I**.

GC column[a]	**Length**	**Inner diameter**	**Film thickness**[a]	**Retention time**[b]
SE30	25 m	0.32 mm	0.5 μm	7.3 min
PAS1701	25 m	0.32 mm	0.25 μm	9.5 min
DB-1701P	30 m	0.32 mm	0.25 μm	10.0 min
BPX-10[c]	30 m	0.25 mm	0.25 μm	11.2 min

.

LC column	**Length**	**Inner diameter**	**Chromatographic conditions**	**Retention time**[d]
Shodex Rspak DE-413	150 mm	4.6 mm	Eluent: acetic acid in water at pH 2.6; Flow 1.0 ml/min	4.0 min
Hypercarb[c]	50 mm	2.1 mm	Eluent: water (without buffer); Flow 0.2 ml/min	3.5 min

[a] The used chromatographic conditions equivalent for the different columns, with a column pressure of 40 kPa.
[b] The retention time of the analyte, 2,3-dibromopropionamide, and that of the brominated internal standard, 2,3-dibromo($^{13}C_3$)propionamide, were identical.
[c] Column used for measurements in the present thesis studies.
[d] The retention time of acrylamide and that of the internal standard, ($^{13}C_3$)acrylamide were identical. Retention time for column without precolumn given.

In addition to the proceedings above, there were comparisons with an external laboratory, National Food Administration (NFA), Uppsala, Sweden, giving conclusive results.

According to earlier estimations (Bergmark, 1997; Törnqvist, *et al*., 1998), the background Hb-adduct level of acrylamide could correspond to a relatively high uptake of acrylamide (about 80-100 μg/day). Food could be the source/one major source explaining this observed high background level of acrylamide in humans. The levels of acrylamide in food was compatible with results obtained with this adduct method.

Since then the occurrence of acrylamide in food was discovered, many laboratories around the world have confirmed the findings. The instrumental techniques for the determination of the acrylamide monomer, is the same, independent of the origin of the sample. What differs is the extraction of the sample and sample treatment, which is dependent on the source of the sample. Used methods, mainly for analysis in foods, have been reviewed (Castle & Eriksson, 2005; Wenzl, de la Calle & Anklam, 2003; and Zhang, Zhang & Zhang, 2005).

A strong way, of performing identification of an analyte is to use completely different analytical methods. Other laboratories later used this concept as well, for identifying acrylamide in food (Ahn, *et al*., 2002; Yoshida, *et al*., 2002; Ono, *et al*., 2003; Jezussek & Schieberle, 2003b; Ciesarová, *et al*., 2004; Hoenicke, *et al*., 2004b). The use of two independent methods is valuable especially during method development and validation, but the extra cost of analysis using two different methods is not affordable for routine analysis and is not needed once a method is validated and the scope (applicability) of a method is proven.

6 Analytical methods for analysis of acrylamide in foods and air (Paper I-V)

In principle, all analysis of organic pollutants follows the same procedure, consisting of extraction, purification, concentration and instrumental analysis. At AnalyCen, we have used both GC-MS and LC-MS/MS methodology for acrylamide analysis. In practice, the LC-MS/MS method is used for routine analysis and the GC-MS method is used for confirmatory reasons. Our two methods have shown very similar result, corresponding to 5-5000 μg/kg food) the calibration line was linear (r^2=0.9999) and LC-MS/MS values were found to be 0.99 of the GC-MS values with the two different methods used in our laboratory. Our GC-MS method has a limit of detection (LOD) of 5 μg/kg (**Paper I).** Our LC-MS/MS method had a LOD of 10 μg/kg (**Paper I and II)**, which was later improved to 3 μg/kg (**Paper III-V)**. Since the occurrence of acrylamide in food products now is scientifically accepted, it is no longer common to use two independent methods for verifying acrylamide results. Still, samples occur where the separation in the LC-MS/MS method is not sufficient. In these cases, to be able to get a quick answer on the content without performing extensive method developments, the use of the GC-MS method is necessary.

In this chapter I will describe the methods used in our laboratory for acrylamide analysis in foods in more detail and compare with what others have done.

A flow-scheme of used methods in Chapter 6 in Figure 6.1.

6.1 Extraction of foods with water (Paper I-V)

The high water-solubility of acrylamide (see Chapter 4.1) means that extraction of foods using plain water is very effective. We have used water extraction at a mass ratio of about one part fine grinded sample plus ten parts water at room temperature. The extract is not pH adjusted. Since the buffer capacity of destilled and de-ionized water is very low, the water extract will have about the same pH as the sample. For food products it is usually in the range of 5-7. At AnalyCen there have been a need to use other extraction solvents only in the analysis of oil and fats (including cacao butter). The extraction then has involved dissolving 1 gram of fat/oil in 10 mL *n*-hexane, addition of internal standard and extraction one time with 10 mL water. The water phase is then analyzed in the same way as for other food materials.

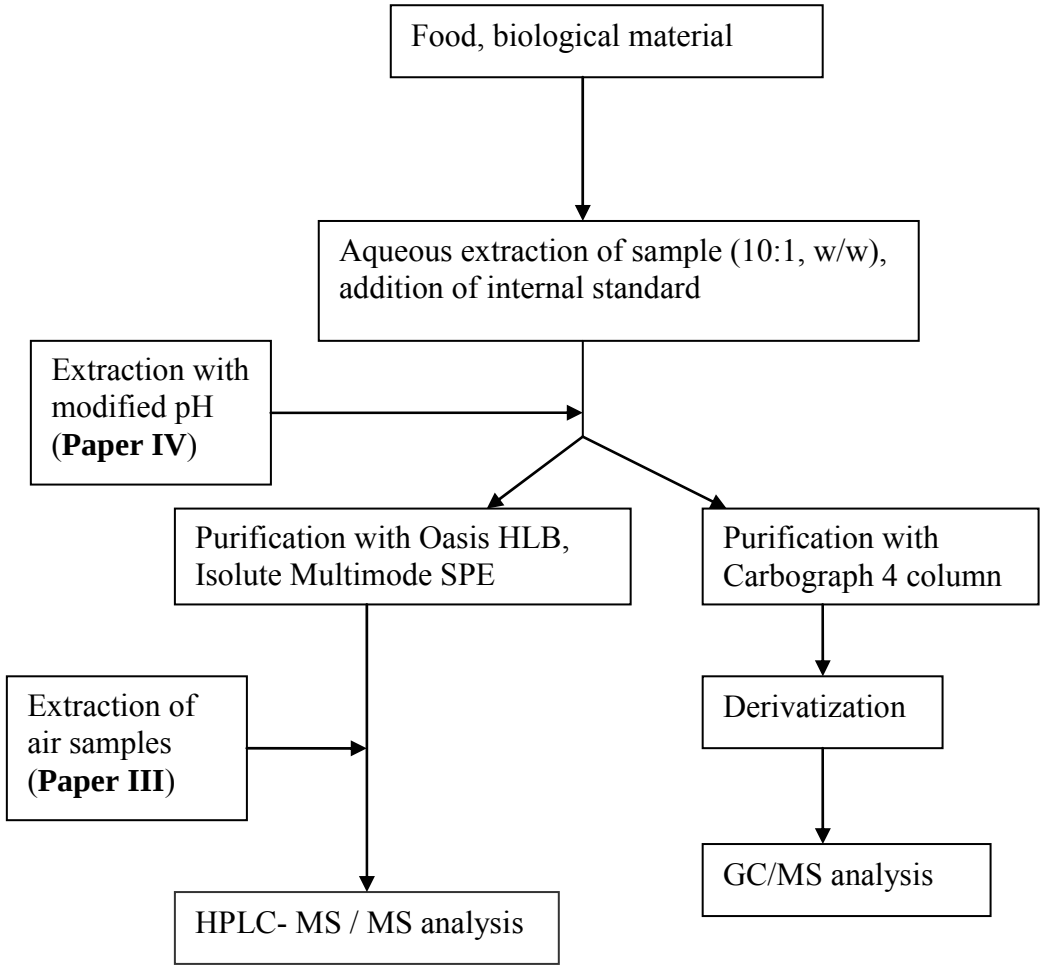

Figure 6.1 Summary of used analytical methods in this thesis for measurement of acrylamide,

Most laboratories, later performing acrylamide analysis has used water extraction.

Effect of other extraction techniques on acrylamide content in food, is described in Chapter 9, **Paper IV**.

6.1.1 Purification of extracts (Paper I-V)

In general, purification of water extracts for acrylamide analysis has followed three principles: a) purification with SPE columns; b) chemical purification, i.e. deproteination; c) no purification before derivatization. We have exclusively used SPE technology.

We have chosen to use SPE techniques, since this is a way to minimize the use of chemicals and it is a technique, which is easy to automatize. This is necessary as a large number of samples are analysed in our laboratory. SPE are used for

both our GC and LC methods, but with different working principles. We have used graphitized carbon black column (Carbograph 4) for the GC-MS **(Paper I)** before derivatization of the extract, and a mixed-mode containing non-polar (C_{18}), strong cation exchange ($-SO_3^-$) and strong anion exchange ($-NR_3^+$) functional groups column (Isolute® Multimode) for the HPLC-MS/MS method (**Paper I-V**), and in combination with a polar and non-polar phase [poly(divenylbenzene-co-N-vinylpyrrolidine] column (OASIS® HLB) to have an even more pure extract in **Paper III-V.**

Different SPE materials for the GC and HPLC methods are used because we found it necessary in the verification procedure for acrylamide to use different types of SPE materials with different effects (cf. 5.2). The effect on separation of Carbograph 4, which contains graphitized carbon black, is in the HPLC method obtained by using an analytical column of a similar material. This technique worked well, and was applied in the further work.

In the work concerning acrylamide analysis in food that has followed at other laboratories, different kinds of SPE columns for purification of the extracts have been used. These are: a) graphitised carbon, b) ion-exchanger and c) mixed materials. In most cases, the choice of any particular clean-up SPE cartridges has been based on trial and error, i.e. what is available in house and experience at the laboratory of different types of SPE materials. When interference is observed for a particular type of food, many authors have tried a number of different SPEs until the problem has been resolved. Consequently, a large number of SPE materials have been reported to be used in acrylamide analysis, as given in Table 6.1.

In many cases, the SPE clean-up step has been combined with a molecular size cut-off filter to remove larger molecules. Some laboratories, for instance ours, do not use them any longer, since they from time to time have been contaminated by acrylamide monomer (Fohgelberg, *et al*., 2005).

6.2 Extraction of air samples (Paper III)

Acrylamide in air has been measured after sampling with standard (150/75 mg) XAD-7 tube connected to a personal sampling pump. Immediately after sampling, the two ends of the tube were sealed with plastic covers, and put in a refrigerator at 4 ºC until extracted. During the extraction, the front (150 mg) and back (75 mg) sections of the XAD-7 tube were placed in separate extraction tubes, and extracted with 1 mL 5% methanol in water (OSHA Method PV2004, 1991) containing 0.01 µg/mL internal standard (same internal standard as for food analysis). The tube was shaken for 30 minutes and then centrifuged at 2500 rpm for 5 minutes. The supernatant was transferred to a HPLC vial, stored at 4 ºC until time for analysis with HPLC-MS/MS as described below.

Table 6.1 Used SPE column materials by different laboratories performing acrylamide analysis.

Carbograph 4	Graphitized carbon black (**Paper I).**
Isolute® Multimode	Mixed-mode containing non-polar (C_{18}), strong cation exchange (-SO_3^-) and strong anion exchange (-NR_3^+) functional groups (**Paper I - V;** Rosén & Hellenäs, 2002; Ahn, *et al*., 2002; Gutsche, *et al*., 2002; Yoshida, *et al*., 2002; Ono, *et al*., 2003; Riediker & Stadler, 2003; Shih, *et al*., 2004; Delatour, *et al*., 2004; Granby & Fagt, 2004).
OASIS® HLB	Polar and non-polar phase [poly(divenylbenzene-co-N-vinylpyrrolidine] (**Paper III-V;** Leung, *et al*., 2003; Roach, *et al*., 2003; Andrzejewski, *et al*., 2004; Gökmen, *et al*., 2005; Becalski, *et al*., 2004; Tsutsumiuchi, *et al*., 2004; Mestdagh, *et al*., 2004; Young, *et al*., 2004; Şenyuva & Gökmen, 2005b).
AccuBOND II SCX	Strong benzene-sulphonic acid-based sorbent cation-exchanger with significant non-polar secondary interactions (Riediker & Stadler, 2003).
Bond Elut AccuCAT	Mixed-mode sorbent comprised of sulphonic acid and quaternary amine (Leung, *et al*., 2003; Roach, *et al*., 2003; Takatsuki, *et al*., 2003; Andrzejewski, *et al*., 2004; Murkovic, 2004; Becalski, *et al*., 2004; Tsutsumiuchi, *et al*., 2004; Mestdagh, *et al*., 2004).
Bond Elut C18	Hydrophobic silica-based sorbent (Takatsuki, *et al*., 2003).
Bond Elut Jr-PSA	Irregularly shaped acid-washed silica with ethylenediamine-N-propyl bonded functional group (Takatsuki, *et al*., 2003).
ENVI-Carb	Graphitised nonporous carbon (Becalski, *et al*., 2003).
Isolute® MF C18	Monofunctional C_{18} silane, covalently bonded to the surface of a silica particle (Gutsche, *et al*., 2002).
OASIS® MAX	Strong anion-exchange dimethylbutylamine groups on the surface of HLB (Becalski, *et al*., 2003).
OASIS® MCX	Strong cation-exchange sulphonic acid groups on the surface of HLB (Becalski, *et al*., 2003; Young, *et al*., 2004).
Sep-Pak® Plus C18	Silica-based bounded phase with strong hydrophobicity (Eberhardt II, *et al*., 2005).
strata™-X-C	Polymeric resin functionalised with polar and strong cation exchange groups (Peng, *et al*., 2003).

6.3 Methodology for GC-MS analysis (Paper I)

Bromination of acrylamide in samples is the most common derivatization procedure when the analysis is performed by GC-MS. This methodology has been used for all kinds of matrices, and has been used in **Paper I**.

6.3.1 Derivatization by bromination

Analysis of acrylamide using bromination and GC determination was relatively commonly applied and advanced technique even before acrylamide was discovered in heated foods, because of the need to test drinking water, discharged water, crops and other matrices for acrylamide (Lande, Bosch & Howard, 1979; Castle, Campos & Gilbert, 1991; Castle, 1993; Bologna, *et al*., 1999). Bromination offers an analyte that is much easier to analyze at trace levels than acrylamide itself. In **Paper I**, the sample extract after Carbograph 4 SPE column were derivatized through bromination by using potassium bromide (7.5 g), hydrobromic acid (acidification to pH 1-3), and saturated bromine water (10 mL) according to the method by Castle *et al*. (Castle, Campos & Gilbert, 1991; Castle, 1993). The sample was kept at 4 °C over-night and the excess bromine decomposed by adding sodium thiosulphate (1 M) drop-wise until the yellow color disappeared. Sodium sulphate (15 g) was added, and the solution extracted with ethyl acetate: hexane (2×20 mL, 1:4 (v/v)). The two-pooled organic fractions were dried and evaporated on a rotary evaporator to about 200 µL. The analyzed product of bromination was 2,3-dibromopropionamide (eq. 1).

$$Br_2 + H_2C{=}CH\text{-}CONH_2 \rightarrow H_2CBr\text{-}CHBr\text{-}CONH_2 \quad (eq.\ 1)$$

High concentrations of acid [H^+] and bromide [Br^-] are required to suppress the loss by dissociation of free elemental bromine, Br_2, which is the active agent. The 4 °C over-night long reaction time has its history in that it was required due to the internal standard methacrylamide or *N,N*-dimethylacrylamide, which were in use before MS-techniques were common (when GC-electron capture (GC-EC) and other detection techniques were used). These compounds had a lover reaction rate with bromine than acrylamide itself. Today, when GC-MS or GC-MS/MS techniques are available, isotopic substituted acrylamide is always recommended. With the use of isotopic substituted acrylamide as the internal standard, the reaction time can be shortened to one or two hours (Nemoto, *et al*., 2002; Gertz & Klostermann, 2002; Ono, *et al*., 2003; Hamlet, Jayratne & Sadd, 2004). We have however, kept the longer reaction time since in the daily laboratory work with these analyses, it is practically to have the bromination incubation over night.

When we were performing GC-MS analysis of 2,3-dibromopropionamide, two µL of the samples were injected splitless, with an injector temperature of

250 °C. The temperature program for the GC was, with columns given in Table 5.2: isothermal for 1 min at 65 °C, then increased at a rate of 15 °C /min to 250 °C, and isothermal for 10 min. The analysis was performed using electron ionization (70 eV) and selected ion monitoring. The ions monitored for identification of the analyte, 2,3-dibromopropionamide, were *m/z* 152 $[C_3H_5{}^{81}BrON]^+$ (100 %), 150 $[C_3H_5{}^{79}BrON]^+$ (100 %) and 106 $[C_2H_3{}^{79}Br]^+$ (65 – 70 %) using *m/z* 150 for quantification. The ions monitored for identification of the internal standard, brominated to 2,3-dibromo($^{13}C_3$)propionamide, were *m/z* 155 $[^{13}C_3H_5{}^{81}BrON]^+$ (100 %) and 110 $[^{13}C_2H_3{}^{81}Br]^+$ (65 - 70 %) using *m/z* 155 for quantification (a variation of ±10 % in ratio between fragment ions were allowed for identification).

Quantification was performed by comparison with a calibration curve (0.5 - 50 µg/L water, corresponding to 5 – 500 µg/kg) for 1 g sample and 10 mL water extract. Samples with higher concentrations than 500 µg/kg of acrylamide were diluted by a factor of 6 in the first step when food was mixed with water. Recovery tests were repeatedly performed by quantification of acrylamide in different (both raw and heated) foodstuffs before and after the addition of acrylamide.

The GC columns have large variations in polarity, depending on the way of derivatization for acrylamide analysis, or if un-derivatized acrylamide has been analyzed. Examples of useful columns for brominated acrylamide are given in Table 5.2.

6.4 Methodology for LC-MS/MS analysis (Paper I-V)

Extraction and purification follows the same steps as for the GC methods, and the earlier described methods are also valid here for purification of the extracts.

6.4.1 Choice of LC column

LC-MS/MS (ESI+) was used for analysis of the samples in **Paper I–V,** by using Hypercarb®column at ambient temperature. Deionized water was used as the mobile phase, with a flow rate of 0.2 mL/min for 6.1 min (analytes recorded), washing with 80 % aqueous acetonitrile (4 min at a flow rate of 0.4 mL/ min) followed by reconditioning with water (0.2 mL/min, 10 min). In order to obtain reproducible results, it was found to be important to include a cleaning step after each injection. This was especially important when handling samples of unknown origin, otherwise, irregularly occurring late peaks and interferences could spoil the analysis.

As with the SPE clean-up, different laboratories have used a number of different stationary-phases for analytical LC-columns. This has been done in order to obtain separation of acrylamide from other co-extractives. Examples of such

stationary-phases are: a) graphitic carbon, b) hydrophilic end-capped C18, c) ion-exchanger, d) others. A number of LC-columns, which have been utilized for acrylamide analysis, are presented in table 6.2.

Interestingly, it has been observed that independently of the LC-columns used (when used properly) no large variation of measured acrylamide levels has been obtained. The most frequently used column type until today has been graphitic carbon (also used by us in **Paper I-V**).

When analysing acrylamide in samples with LC-MS/MS the quantification were performed through comparison of peak areas (as for GC-MS) with a calibration curve with 5 concentrations (1 - 500 µg/L water, corresponding to 10 – 5000 µg/kg). The repeatability, recovery and reproducibility of the LC-MS/MS method were evaluated and the method was compared with the GC-MS method in the tested aspects, and both methods became accredited at the same time (SWEDAC, 2002)(see also **Paper I**).

6.4.2 Instrumental LC-MS/MS conditions

Samples, presented in **Paper I-V** that have been analyzed by LC-MS/MS with electrospray ionization in positive ion mode (ESI+). This has then become the normal way of performing analysis with LC-MS/MS (see **Paper I-V**; Rosén & Hellenäs, 2002; Gutsche, *et al*., 2002; Ahn, *et al*., 2002; Ono, *et al*., 2003; Leung, *et al*., 2003; Hartig, *et al*., 2003; Roach, *et al*., 2003; Becalski, *et al*., 2003; Riediker & Stadler, 2003; Ahn & Castle, 2003; Andrzejewski, *et al*., 2004; Shih, *et al*., 2004; Hoenicke, *et al*., 2004b; Granby & Fagt, 2004). The instrumental parameters are relatively dependent on the used equipment. The instrumental settings for our LC-MS/MS equipments (three different instrumentations) when optimized for acrylamide analysis are presented in Table 6.3.

Table 6.2 Different types of used stationary-phases in analytical columns for LC analysis of acrylamide.

Hypercarb®	100% porous graphitic carbon (**Paper I-V**; Rosén & Hellenäs, 2002; Gutsche, *et al*., 2002; Leung, *et al*., 2003; Hartig, *et al*., 2003; Becalski, *et al*., 2003; McHale, *et al*., 2003; Shih, *et al*., 2004; Granby & Fagt, 2004; Tsutsumiuchi, *et al*., 2004).
Aquasil C18	C18 with hydrophilic end capping (Becalski, *et al*., 2004).
Aminex HPX-87H	Polystyrene divinylbenzene resin, separation by ion moderated part chromatography technique (Paleologos & Kontominas, 2005).
Atlantis™ dC18	Difunctionally bonded silica columns (Yoshida, *et al*., 2002; Ono, *et al*., 2003; Gökmen, *et al*., 2005; Young, *et al*., 2004; Mestdagh, *et al*., 2004).
Atlantis™ HILIC	Hydrophilic Interaction Chromatography column (Gökmen, *et al*., 2004).
Inertsil ODS-3	ODS phase and end capping (Şenyuva & Gökmen, 2005a, 2005b).
IonPac® ICE-AS1	Cross-linked styrene/divinylbenzene resin that is functionalized with sulfonate groups (Höfler, *et al*., 2002; Dionex, 2002, Cavalli, *et al*., 2002).
LiChrosphere®CN	Propylcyano-modified silica gel phase (Hartig, *et al*., 2003).
LiChrosphere®100 CN	Propylcyano modified silica gel phase with spherical particles (Hoenicke, *et al*., 2004b).
Luna® C-18	C18, end-capped (Hartig, *et al*., 2003; Wagner & Flynn, 2003; Calbiani, *et al*., 2004).
Luna® Phenyl-Hexyl	Phenyl with Hexyl (C6) linker bonded to Luna silica surface, end-capped (Jezussek & Schieberle, 2003a,b).
Mightysil RP-18 GP	C18, end-capped (Inoue, *et al*., 2003).
PrimeSphere™ C18-HC	C18, hydrophilic end capping (Ahn, *et al*., 2002).
Shodex ™ RSpak DE-613	Polymeric Reverse Phase of Polymethacrylate (Riediker & Stadler, 2003; Delatour, *et al*., 2004).
Shodex™ Sugar KS-801	Counter ion Na^+. Size exclusion chromatography is the dominant mode of the column (Terada & Tamura, 2003).
Synergi™ Hydro-RP	A C18 with polar end capping (Peng, *et al*., 2003; Roach, *et al*., 2003; Andrzejewski, *et al*., 2004).
Synergi™ Polar-RP	An ether-linked phenyl with polar end capping (Brandl, *et al*., 2002; Murkovic, 2004).
YMC-Pack™ ODS-AQ™	C18, hydrophilic end capping (Zyzak, *et al*., 2003; Eberhardt II, *et al*., 2005).

Even though quadrupole LC-MS/MS analysis are known to be one of the more "secure" methodologies to use, there are a lot of troublesome stages when analysing acrylamide.

- Reversed-phase mode and the chromatographic retention of acrylamide can be quite poor. We have used active carbon column in order to obtain sufficient retention time.
- The desolvation chamber temperature of the MS can be set as high as 280 to 350 ºC, as we have used, although the drying stream will not get so hot because of evaporative cooling. The evaporation is not as effective as thought.
- The ionisation source itself can be set at 125 ºC or at higher temperature, as we have used. This implies a risk therefore that in the analysis of food extracts or of model systems, co-eluting precursors or irreversibly bound acrylamide may break down or be released in the hot regions of the mass spectrometer and give a false acrylamide signal.
- The applied clean-up procedure for the sample extract and the LC column resolution parameters is of importance for successful analysis. Relying on the resolution power of the mass spectrometer is not adequate, i.e. it is more important with the cleaning step when using LC compared to GC.
- Most modern LC-MS/MS instruments perform relatively poorly at molecular weights below 100 unless tuned specifically for low mass/charge ratio ions.

To our knowledge, the way we have designed the GC-MS and LC-MS/MS methods, we have resolved these problems. One of the main ways to demonstrate this, is to take part in proficiency tests, were our results so far have been performed in an excellent way, see Table 6.4.

Table 6.3 Optimized settings for acrylamide on three instruments (Micromass) at AnalyCen.

Parameter	Ultima, old	Ultima, new	Premier
Polarity	ES+	ES+	ES+
Capillary (kV)	3.2	1	1
Hex 1 (V)	0.2	25	N/A
Aperture (V)	0.2	0	N/A
Hex 2 (V)	0.2	1	N/A
Extractor (V)	N/A	N/A	5
RF lens (V)	N/A	N/A	0
Source temperature (ºC)	125	140	120
Desolvation temperature (ºC)	350	400	450
Cone gas flow (L/Hr)	210	210	23
Desolvation gas flow (L/Hr)	650	650	600
LM 1 resolution	13	12	13
HM 1 resolution	13	12	13
Ion energy 1	1	1	0.5
Entrance	-5	-4	-2
Exit	1	3	1
LM 2 resolution	13	8	13
HM 2 resolution	13	8	13
Ion energy 2	1	1	0.5
Multiplier (V)	650	650	650
Ion 1	72.00>53.90	72.00>53.90	72.10>54.30
Cone (V)	50	40	23
Collisions energy	16	16	12
Ion 2	72.00>54.90	72.00>54.90	72.10>55.20
Cone (V)	50	40	22
Collisions energy	11	10	9
Ion 3	75.00>57.90	75.00>57.90	75.00>58.00
Cone (V)	50	40	23
Collisions energy	11	10	9

6.5 Quality assurance

6.5.1 Performance of the methods

Control/reference samples and proficiency testing of acrylamide are available from several organisations (Wenzl, *et al*., 2004; Owen, *et al*., 2005; Klaffke, *et al*., 2005). This can be a helpful tool in the quality assurance of analytical methods. This ensures that laboratories are producing similar results, and as help when laboratories are working with quality assurance, such as the EN ISO 17025 norm. How laboratories have succeeded in the proficiency tests is normally shown by the z-score. The z-score is defined as

$z = (x\text{-}asv)/\sigma$

were x is the measurement of analyte concentration in the test material, asv is the assigned value, and σ is the target value for the standard deviation (Owen, *et al*., 2005). Our utilized LC-MS/MS method have in proficiency tests shown to be very reliable and robust, Table 6.4, compared to used limits for z, which is $z \leq 2.0$ satisfactory; $2.0 < z < 3.0$ questionable; $z \geq 3.0$ unsatisfactory, since we have had no value outside ±1, and with a mean value of 0.2.

Table 6.4. Summary of proficiency tests from the organizations Food Analysis Performance Assessment Scheme (FAPAS) and European Commission's Directorate General Joint Research Centre (JRC), in which our laboratory has participated.

Type of sample	**Assigned value** (μg/kg)	**z-Score**
Crisp bread	1213	-0.7
Potato Crisps	167	-0.6
Breakfast cereals	109	0.0
Coffee	312	-0.6
Crisp bread	707	-0.6
Breakfast cereals	95	-0.6
Oven Chips	1843	0.6
Coffee	174	0.1

Type of sample	**Assigned value** (μg/kg)	**z-Score**
Baby Rusks	711	0.3
Breakfast cereals	70	-0.2
Butter cookies	150	-0.8
Crisp bread	57	-1.0
Bread extract	116*	-0.5
Crisp bread 1	46	-0.4
Crisp bread 2	498	0.0
Crisp bread 3	414	0.0
Coffee extract	858	0.6
Coffee surrogate	1334	0.0
Roasted coffee	258	0.0
	Tot MEAN	**-0.2**

* μg/L

6.5.2 Ion abundance criteria for LC-MSMS

The ions measured on LC-MS/MS at ESI+ mode, which we used in **Paper I-V** were *m/z* 72 (MH^+), 55 ($MH^+ \rightarrow [MH\text{-}NH_3]^+$) and 27 ($MH^+ \rightarrow [C_2H_3]^+$). For the ($^{13}C_3$)acrylamide internal standard the corresponding ions are m/*z* 75, 58 and 29. These ions have in general been in use by others, performing analysis with similar MS/MS conditions for analysis of acrylamide.

In general, a typical criterion for identification is that the relative abundance values should agree to ±10 % for acrylamide to be detected (Roach, *et al*., 2003; Andrzejewski, *et al*., 2004). In a similar way, acrylamide in food is confirmed if at least two positive selective-reaction monitoring (SRM) responses are obtained with matching ion ratios within an acceptable tolerance (mean ±10-20 %) compared to the ratios obtained from acrylamide standards, which fulfil the criteria according to EU Directive 2002/657/EC (EU Commission 2002c; Riediker & Stadler, 2003). Another criteria which has been used is to examine the full mass spectrum obtained, which is always performed on unknown substances, when deciding which ions to use for routine quantitative analysis.

6.6 Quantitative aspects

The limit of quantification (LOQ) and limit of detection (LOD) obtained by most laboratories is still (compared to our **Paper I**) within the same magnitude as obtained by us, independent of which LC-MS method they have used. This means that typically, LOD values spans from 3 to 20 μg/kg and that LOQ values spans from 10 to 50 μg/kg, and the analysis is linear over the range 10 to 10,000 μg/kg (with some variations).

6.7 Complementary and new analytical methods

6.7.1 Extraction

Some laboratories have since **Paper I** was published**,** tested other alternatives for extraction, for example using hot water for one to two hours did not seem to improve the recovery (Owen, *et al*., 2005). However, different laboratories have found that extraction of fatty matrices such as chocolate or peanut butter are improved when using hot water to promote dispersion and effective extraction (Ahn, *et al*., 2002; Zyzak, *et al*., 2003; Gutsche, Weißhaar, & Buhlert, 2002). Alternatively, room temperature extraction using a binary mixture of water and an organic solvent such as ethylene dichloride to break-up and remove the fat phase has been shown to be very effective (Zyzak, *et al*., 2003). In some laboratories where the use of halogenated solvents is discouraged or prohibited, other organic solvents, including hexane, have been used for defatting the sample prior to water extraction (Gutsche,

Weißhaar, & Buhlert, 2002; Vattem & Shetty, 2003; Hartig *et al*., 2003). The use of solvents such as hexane should be beneficial in accordance to the low solubility for acrylamide in unpolar solvents (see Table 4.1).

Analysis with older instrumentation often requires concentration of extracts to be able to obtain low detection limits; this implies that the extraction is performed with organic solvents, which have a high solubility of acrylamide.

Alternative ways of performing extraction is to utilize Accelerated Solvent Extraction (ASE®) technique, which has been used in combination with LC-MS/MS (Brandl, *et al*., 2002). This extraction method has also been applied with LC-MS in positive-ESI mode and LC with UV detection (Höfler, *et al*., 2002).

Acrylamide concentrations in air could be measured after sampling and extraction. Sampling could be done by using impinger flasks with water (Skelly, & Husser, 1978), phosphoric acid treated, Flusin-F (Suzuki, & Suzumura, 1977), Sep-Pak Plus PS-2 cartridge (Koga, *et al*., 1998), silica gel (OSHA Method 21, 1980; Hills, & Greife, 1986; Pantusa, *et al*., 2002) or by XAD-7 adsorbent, which were used in this thesis (**Paper III**). The extraction from the adsorbent, have to be performed in a way that no polymerization of acrylamide occurs.

6.7.2 Purification

In order to remove proteins from samples chemical deproteination by using Carrez solutions I and II (based on $K_4[Fe(CN)_6]$ and $ZnSO_4$-salt solutions) has been used in combination prior to GC and LC analysis by many laboratories (Gutsche, *et al*., 2002; Gertz & Klostermann, 2002; Hartig, *et al.*, 2003; Gökmen, *et al*., 2004; Delatour, *et al*., 2004; Hoenicke, *et al*., 2004b; Şenyuva & Gökmen, 2005a, 2005b). This technique has not been applied in our laboratory in the acrylamide analysis.

6.7.3 Other GC methods

Analysis with GC can be performed either after derivatization with bromine or directly (Biedermann, *et al*., 2002a; Tateo & Bononi, 2003; Matthäus, *et al*., 2004; Ciesarová, *et al*., 2004; Al-Dmoor, Humeid & Alawi, 2004; Al-Dmoor 2005; Bononi, *et al*., 2005). GC-MS/MS analysis of both 2,3-dibromopropanamide after bromination or without derivatization of acrylamide has been performed (Hamlet, Jayratne & Sadd, 2004; Hoenicke, *et al*., 2004b).

An alternative (not used in this thesis) is to form the bromine *in situ* by using trace amounts of potassium bromate when doing bromination of acrylamide (Nemoto, *et al*., 2002).

In combination with headspace solid-phase microextraction (HSPME), acrylamide can be silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to form the volatile *N,O*-bis(trimethylsilyl)acrylamide (BTMSA). This methodology together with ion-trap mass spectrometry gave a detection limit of about 1 µg/kg when analyzing cereal matrices for acrylamide (Lagalante & Felter, 2004).

6.7.4 Other LC methods

LC-UV has been used to analyze acrylamide content in potato products and instant noodles in which levels can be rather high and sensitivity is not a major issue. By first performing bromination (as for GC), the selectivity increases a lot and a lower detection limit (µg/kg level) can be reached for some food products (Ozawa, *et al*., 1998). To compensate for the measurement at a universal wavelength (cf. 4.4.2) and lack of specific detection, column-switching techniques have been used to get a better separation (Terada & Tamura 2003). A drawback with this technique is that isotopic substituted acrylamide cannot be used as an internal standard. For French fries and other foods, LC-UV at low wavelengths gave compatible results to LC-MS measurements (Höfler, *et al*., 2002; Dionex, 2002, Cavalli, *et al*., 2002; Peng, *et al*., 2003; Gökmen, *et al*., 2005; Paleologos & Kontominas, 2005).

To compare the use of LC-MS triple-quadrupole mass spectrometers with single-stage instruments (LC-MS/MS vs. LC-MS), LC-MS instruments are less expensive and have not usually had sufficient sensitivity to conduct acrylamide analysis on water extracts of foods unless a degree of pre-concentration is used. To be able to reach the same detection limit as LC-MS/MS, different techniques have been developed. These include column switching to an ESI mode MS instrument (Inoue, *et al*., 2003; Takatsuki, *et al*., 2003). An alternative is to change to an organic solvent or by use SPE columns for concentration (not only purification). Both in combination with a concentrating of the sample extract to a small volume, and analysis using LC-MS with atmospheric pressure chemical ionization (APCI) (Zyzak, *et al*., 2003; Eberhardt II, *et al*., 2005). Another alternative is the usage of electrospray in the positive-ion mode and selected-ion recording (Young, *et al*., 2004). For other laboratories, the sensitivity of their LC-MS equipment is sufficiently low for their analysis without doing this type of method development (Murkovic, 2004; Şenyuva & Gökmen, 2005a, 2005b).

A complementary form of derivatization together with LC-MS is with 2-mercaptobenzoic acid, again with positive-ion ESI, which is an interesting alternative (Jezussek & Schieberle, 2003a, 2003b). The method using derivatization with the sulphur nucleophiles 2-mercaptobenzoic acid looses

the apparent simplicity of direct acrylamide analysis but in principle gives some advantages. First, by making the molecule less polar it improves the retention on the LC column and increases the potential for separation. Second, derivatization with 2-mercaptobenzoic acid offers the possibility of a sensitive LC-UV method for routine analysis, e.g. factory quality control, or even LC-fluorescence analysis if a fluorescent sulphur nucleophile were to be used in place of 2-mercaptobenzoic acid.

Ion trap, which is an alternative to the quadrupole instruments, has the problem of over-saturation of the trap with ions, and has been considered to be less reliable in quantitative analysis. Even though, comparable results for acrylamide analysis have been obtained with ion trap MS/MS compared to quadrupole MS/MS analysis (Tsutsumiuchi, *et al*., 2004).

6.7.5 Microemulsion electrokinetic chromatography

Capillary electrophoresis, like microemulsion electrokinetic chromatography (MEEKC) have been used, and tried for analysis of acrylamide in French fries. It was linear in concentration 1.25-125 mg/L, and with a detection limit of 0.7 mg/L (Bermudo, *et al*., 2004).

6.7.6 Quartz microbalance sensor

Several macrocycles of the Hunter-Vögtle type (i.e. a type of tetralactam macrocycles) can serve as a highly sensitive and selective sensor-active layers in quartz microbalances. They have also been identified as superior host compounds for the detection of small amounts of acrylamide, and can give a detection limit of 10 µg/kg. Other related compounds like acrylic acid, propionamide or propionic acid showed no or significantly lower affinities to the macrocycles in the actual concentration range (Kleefisch, *et al*., 2004).

6.7.7 Future

For routine purpose, different types of instruments and techniques can be used, which fulfil the demands on separation and sensitivity. LC-MS/MS is an expensive technique to use, and the newest LC-MS has sufficient sensitivity for acrylamide analysis. The need for LC-MS/MS will mainly be for identification.

Chemical companies have even started to sell kits for analysis of acrylamide in foods according the Swiss method (Walz & Trinh, 2005).

7 Factors affecting acrylamide content (Paper I-IV)

7.1 Our experimental studies, (Paper I-IV)

We have performed experiments with food to study determinants of acrylamide formation and experiments with other biological material to control if formation of acrylamide only occurs when heating/drying food products, or if it is a general process for similar biological materials. Furthermore we have performed air sampling from cooking and drying experiments to analyze if some of the formed acrylamide is getting volatile. The factors, which we have studied in experiments, include temperature, pH, antioxidants, amino acids, protein, carbohydrates, radical initiators and their effect on acrylamide formation, with potato as a food model in most experiments. A summary of the results is described in 7.1.1.

7.1.1 Studies on food (Paper I-II)

To identify determinants for the formation of acrylamide in food, we continued our experiments mainly with potato as a food model, as we had shown a high formation of acrylamide in potato. Time and temperature is of great importance in the formation of acrylamide in heat-treatment. In our first work, **Paper I**, we showed that a temperature of 120 ºC is needed for the formation, and that acrylamide is not formed during boiling.

Prolonged heating time decreases the acrylamide content, as shown in **Paper II**, which also has been shown by others (Gutsche, Weißhaar, & Buhlert, 2002; Biedermann & Grob, 2003; Weisshaar, 2004). It has also been shown that acrylamide can be formed at temperatures below 100 ºC (Biedermann & Grob, 2003). We confirmed this in drying experiments at 65 – 130 ºC in the laboratory, **Paper III**.

In a series of experiments, the influence of different factors was studied in homogenized potato (**Paper II**). For potato we found that the highest acrylamide formation was at about pH 8 when heating for 15 minutes in a GC oven at temperatures of 160 and 180 ºC, **Paper II**. It was described that reducing sugars and asparagine are involved in the formation of acrylamide (Mottram, *et al*., 2002; Stadler, *et al*., 2002). By addition of glucose and fructose (35 mmol/kg), the acrylamide formation increased, but the enhancement at 140 mmol/kg glucose was surprisingly lower than expected from 35 mmol/kg result, Figure 7.1A. This reaction is likely because at the lower addition, the reducing sugar, fructose is more effective than glucose, and consequently the limiting factor. At the higher addition of reducing sugars, it is asparagine, which is the limiting factor in the formation. A

general factor affecting the Maillard reactions is pH, were browning reactions having the maximum yield at pH 10 (Ashoor & Zent, 1984), even though we in our experiments had a formation optimum at pH 8, **Paper II**. A summary of some of the addition experiment results from **Paper II** is shown in Figure 7.1.

Addition of asparagine did also lead to an increase of acrylamide formation, Figure 7.1A. Especially when added to frozen potatoes. This is because during low temperature and freezing, the content of reducing sugars in potato increases. A comment upon this is that potatoes should be stored in a way that reducing sugars in potato stay as low as possible, i.e. above 10 ºC, which have been shown by others (Noti, *et al*., 2003; Chuda, *et al*., 2003; Olsson, Svensson & Roslund, 2004; De Wilde, *et al*., 2004, 2005). If glyceraldehyde or glucose-6-phosphate was added to potatoes, it led to an increase, but not as high as for glucose, **Paper II**. Instead, addition of amino acids (concentration at 35 mmol/kg) other than asparagine, i.e. glycine, alanine, lysine, glutamine or glutamic acid), strongly (up to 90%) decreased the formation of acrylamide in potato, Figure 7.1.A (**Paper II).** This shows that these added amino acids competed in reaction with reducing sugars and consequently reduced the amount of formed acrylamide when added in excess.

Addition of the antioxidants ascorbyl palmitate and sodium ascorbate, gave a decrease of acrylamide content which could be explained by lowering the pH or increased binding water. This pronounced at higher levels, but with minor effect at low levels of addition (1.5 mmol/kg). Negligible effect was noticed by adding hydrogen peroxide or the radical initiator benzoyl peroxide, which demonstrates that used oxidants or radicals, are not involved in the formation of acrylamide, Figure 7.1.B.

When we added citric acid or hydrochloric acid, it showed a decrease of acrylamide formation and an increase of the degradation of formed acrylamide.

Fish meat was used as protein source for mixture into foods. The addition of protein decreased the formation of acrylamide by more than the addition itself would explain, i.e. the added protein was effective in decreasing the formation of acrylamide.

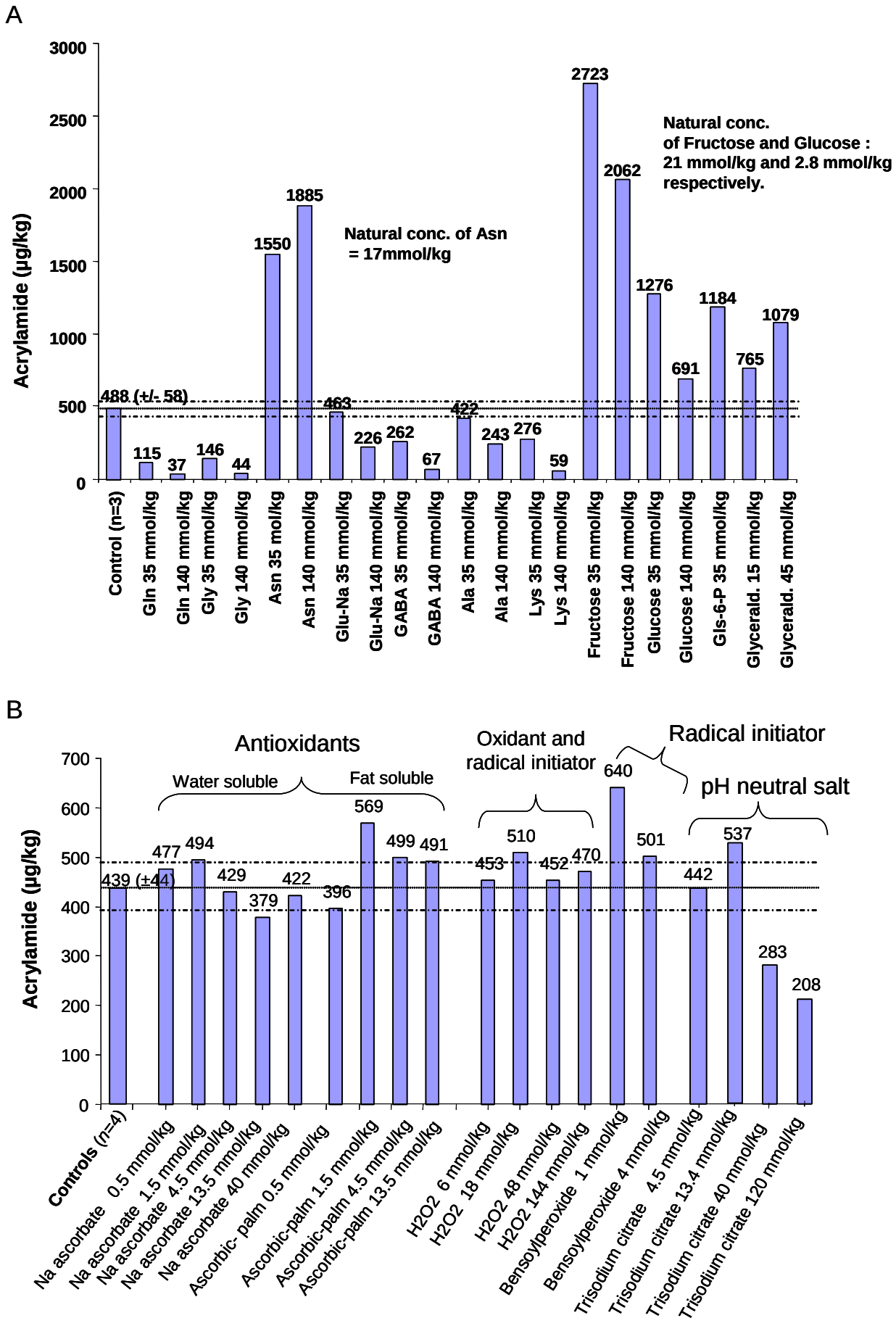

Figure 7.1 Effects of acrylamide content from additives in potato, **Paper II** .

7.1.2 Formation of acrylamide in other biological materials than foods (Paper III)

In our laboratory dry matter determinations are performed in many types of samples, such as agricultural and environmental samples, and we wanted to study whether acrylamide could be formed under these conditions for drying, and subsequently evaporate to the air. In the different methods for drying of various food, feed and environmental samples, temperatures in the range of 65 to 130 ˚C are used.

In all dried analysed samples acrylamide was found, except in dried silage, most likely because the detection limit was not sufficiently low. Control analysis, that acrylamide is really formed during the dry matter determination in some of the non-food samples, and that there is not any acrylamide present in the material before heating, was also performed. The soil, sediment, silage/hay, cow dung/manure samples, have not had any previous heat treatment. Acrylamide was also found in air samples when drying different samples containing biological material at a temperature below 100 ˚C.

Considering the demonstrated formation of acrylamide in sediment/sludge etc, it could be assumed that the pathways of the formation might at least partly differ from the major mechanism identified for acrylamide formation in foods.

7.1.3 Studies on evaporation of acrylamide during heating, Paper III

Even though the vapour pressure of acrylamide is low (see Chapter 4.1) it has been found in air in occupational exposure situations (cf. below). Therefore we thought that it was of interest to analyse the evaporated acrylamide during heating of food and during dry matter determination of food.

For the determination of evaporated acrylamide during heating of food products, we took air samples with a method used to measure occupational exposure to acrylamide (Hills, & Greife, 1986; Pantusa, *et al*., 2002), with small modifications.

A few samples of food products were treated at different temperatures, either simulating cooking/frying or dry matter determinations, to investigate whether a fraction of the acrylamide formed during heating could evaporate and be recovered in samples of air. The food and feed samples were constituted of a mixture of products, like cereal products, meat products, and

so on. The way this study was conducted, gave us no possibility to control the acrylamide content in a specific type of food product in the whole sample, and some of the products in the mixture could have had a low original content of acrylamide. Most of the feeds were pelleted, which involves a slight heat treatment.

After weighing the dry matter samples, the samples from each oven were put together, weighed, mixed and analyzed for acrylamide content with the water extraction methodology (Chapter 6).

In the out-stream air from the ovens, acrylamide was isolated in concentrations from 0.2 ng/m^3 to above 1 μg/m^3, both in heating according to dry matter determinations and the heat treatment simulating conditions for home cooking. In the laboratory, where the well-ventilated ovens are located, the acrylamide content was in the range 1-3 ng/m^3 (n=5). This was in the same order as around the balance, when performing the weighing of the dry matter samples, after the drying procedure. Control samples of air collected in the office area of the laboratory and in the kitchen for cooking experiments, when no food treatment was performed, showed that the levels of acrylamide were below the limit of detection (LOD = 0.1 ng/m^3).

The content of acrylamide has been analyzed in the dried samples, to estimate how large fraction of acrylamide evaporates into the air. This study has clearly shown that a fraction (0.15 – 3.3 % in our experiments) of acrylamide in heated food and other samples evaporates and is found in air when performing house-hold frying, heating in laboratory ovens at 180 ºC and performing dry matter determinations in laboratories at the usual used temperatures for food, feed and environmental samples. The results of this study also indicate that the impact of disappearance of acrylamide through evaporation should be tested in the modelling of acrylamide formation in heating of food. This assumption has to be further investigated.

7.2 Product specific experimental studies performed by others

The above studied factors are included in many investigations of acrylamide formation in specific food products. The summary discussed below mainly concerns western country food products. Similar work is now ongoing all around the world. A lot of research in these fields is performed within the food industry and their partners and are not made public.

7.2.1 Potato products

Potato crisps. Potato cultivar and temperature are important factors for the formation of acrylamide. Blanching (short heat treatment in water systems, to inhibit enzyme activity in raw vegetables) before deep-frying did not affect the amount of acrylamide formed. However the content of reducing sugars determined the level of acrylamide after frying (Weisshaar, 2004; Wicklund, *et al*., in press). Others found an effect of blanching and reducing sugars in amount formed acrylamide (Haase, Matthäus & Vosmann, 2004). Blanching in acidic solutions is an effective way of decreasing acrylamide levels in crisps (Kita, *et al*., 2004). Also linear correlation between acrylamide content of potato chips and their color, represented by redness, was found in the temperature range 120-180 °C, and blanching was found to reduce the acrylamide formation (Pedreschi, *et al*., 2005). A physical tool to decrease the amount of acrylamide formed by up to 95%, is to use low-temperature vacuum frying equipment for crisps (Granda, Moreira & Tichy, 2004).

Fresh potatoes respond to cooling below about 10 °C by increasing the content of reducing sugars, which results in high acrylamide concentration in fried, roasted or baked potato products. The potential for acrylamide formation increases approximately proportionally to the amount of reducing sugars (Noti, *et al*., 2003; Chuda, *et al*., 2003; Olsson, Svensson & Roslund, 2004; De Wilde, *et al*., 2004, 2005). Extraction of surface asparagine and sugars with water, without washing out the starch, is a way to lower the acrylamide content in the final product. Since acrylamide formation increases exponentially towards the end of the frying process, the most important factor to keep acrylamide contents low is the determination of the proper end point of the frying process (Grob, *et al*., 2003). The acrylamide concentration depended on the above factors and on the surface-to-volume ratio of the French fries (Matthäus, *et al*., 2004; Taubert, *et al*., 2004). The oil quality, i.e. oil oxidation and oil hydrolysis do not have any impact on the acrylamide formation (Mestdagh, *et al*., 2004). Addition of citric acid or hydrochloric acid lowers the pH and decreases the acrylamide content in heated food (Jung, Choi & Ju, 2003; Gama-Baumgartner, Grob & Biedermann, 2004). The last-mentioned is comparable to our result, **Paper II**.

Potato croquettes, which are coated with egg and bread crumbs, receive reduced formation of acrylamide, compared to without coating (Fiselier, Grob & Pfefferle, 2004), whose results are similar to what we discovered, when adding fish protein to potato, **Paper II**. In potato, the formation of acrylamide is mainly depending on free asparagine and reducing sugars, limiting factor being the sugar (Biedermann-Brem, *et al*., 2003; Amrein, *et al*., 2004b; Becalski, *et al*., 2004; De Wilde, *et al*., 2005), which is in agreement with our findings in **Paper II**.

7.2.2 Coffee

Coffee has factors affecting acrylamide formation during the roasting procedure. Roasting time, temperature and variety give a variation in content. Extended temperature and time lowered the acrylamide content, since temperature above 240 ºC and time longer than 5 minutes, gave a decrease in acrylamide formation (Bagdonaite & Murkovic, 2004).

7.2.3 Bread

Even though a linear relationship was observed for acrylamide formation in potato, wheat and rye model systems, compared to residual levels of asparagine and reducing sugars (Elmore, *et al*., 2005), there have been a lot of studies aiming to decrease acrylamide formation during baking:

The acrylamide content follows the asparagine content, and the heat treatment with regard to time and temperature. The highest acrylamide content is in the crust, and since the highest asparagine content is not in the starchy endosperm part of the kernel, fiber bread will form higher concentrations of acrylamide (Springer, *et al*., 2003).

In gingerbread, ammonium hydrogen carbonate strongly enhanced acrylamide formation. Both acrylamide concentration and browning intensity are increased with baking time and correlated with each other. The use of sodium hydrogen carbonate as baking agent reduced the acrylamide formation. Reduced levels of asparagine, replacing reducing sugars with sucrose or by adding organic acids, could also lower the acrylamide content (Amrein, *et al*., 2004a). This also occurred to sweet bakeries, like biscuits (Graf, *et al*., 2005). Ammonium hydrogen carbonate also affects acrylamide content in other type of bread (Grothe, *et al*., 2005). Ammonium hydrogen carbonate is an effective amino source in the formation of acrylamide, i.e. acrylamide content increases when ammonium hydrogen carbonate is added, but decreases when sodium hydrogen carbonate is added, which only effect the pH of the product.

One way of reducing free asparagine is to ferment the dough with yeast. Sourdough inhibits the asparagine utilization of the yeast (Fredriksson, *et al*., 2004). Added asparagine, but not fructose has increased the acrylamide content in wheat bread and rye crisp bread. In wheat bread, most of the acrylamide was in the crust (Surdyk, *et al*., 2004; Mustafa, *et al*., 2005; Bråthen & Knutsen, 2005). Addition of glycine reduces the content of acrylamide in cereal and potato products (Bråthen, *et al*., 2005).

In a cracker model, sodium hydrogen carbonate eliminated acrylamide. To a lesser extent, ammonium hydrogen carbonate, cysteine, sodium bisulfite, and ascorbate also enhanced elimination. Citric acid, ferulic acid, and sodium chloride, were found to decrease the amount of acrylamide produced while

having little or no effect on elimination. Asparagine, but not reducing sugar, caused a large increase in acrylamide formation (Levine & Smith, 2005).

7.2.4 Almonds

In almonds acrylamide increases with roasting time and temperature, but temperature have much higher impact on the formation than time. During the roasting procedure sugars are consumed faster and to a larger extent than free asparagine, suggesting that the content of reducing sugars is the critical factor for formation of acrylamide in roasted almonds. Acrylamide was found to decrease in roasted almonds during storage at room temperature (Amrein, *et al*., 2005).

8 Pathways of acrylamide formation

The formation of acrylamide follows different routes in conjunction with the Maillard reactions system in food products, where the asparagine route is the major one for formation of acrylamide, Figure 8.2.

8.1 Asparagine route

We assumed from the beginning of the findings of acrylamide formation in food products assumed that the Maillard reaction was involved (**Paper I**). The discovery that asparagine is involved, was quickly published after our first publication on food (Mottram, Wedzicha & Dodson, 2002; Stadler, *et al*., 2002; Weißhaar & Gutsche, 2002).

Although, thermally allowed decarboxylation and deamination reactions of asparagine can in principle produce acrylamide alone (Yaylayan, Wnorowski & Perez Locas, 2003), the presence of sugars is necessary to effect the conversion of asparagine into acrylamide. Many carbonyl-containing moieties can enhance a similar transformation (Zyzak *et al*, 2003; Becalski, *et al*., 2003; Stadler, *et al*., 2003, 2004).

Concrete evidence, in particular for the formation of the key intermediates in food products, is still lacking (Yayalayan & Stadler, 2005), even though in model studies it has been shown that *N*-glycosyl of asparagine generated 20 times more acrylamide, compared to α-dicarbonyls and the Amadori compound of asparagine. The Strecker alcohol of acrylamide, 3-hydroxypropanamide, generates 20 times less amounts of acrylamide compared with hydroxyacetone. This indicates that α-hydroxy carbonyls are much more efficient than α-dicarbonyls in converting asparagine to acrylamide. Rearrangement of formated azomethine ylide to the decarboxylated Amadori compound is the crucial step (Stadler, *et al*., 2004).

The finding that α-hydroxy carbonyl compounds (such as fructose and glucose) are much more effective than other carbonylic compounds was quite surprising. This can logically only be explained by that the α-hydroxy group must play a central part in the degradation of asparagine by lowering the overall activation energy in the Maillard reaction. We put forward a mechanism (see Figure 8.1, personal com., Per Rydberg), which is based on the key steps in the Maillard reaction. The first step in this reaction is the formation of Schiff bases from asparagine and reducing sugars (intermediate **I**, Figure 8.1). As the pH in most foods are well above the first dissociation constant of asparagine (pK_{a1} = 2.0) (Damodaran, 1996) is consequently the main part of intermediate **I** as a carboxylate ion**,** which enhances the

decarboxylation to intermediate **II**. The decarboxylation reaction (intermediate **I** to **II**) is probably also concerted, which means that this negatively charged α-carbon is directly transferred for removing the hydrogen at the α-hydroxy group (see intermediate **II**) which lowers the activation energy for this reaction. Furthermore, the resulting α-hydroxy anion (a strong base within the molecule) will now be able to remove the acidic α-hydrogen (to the amide) in a six member ring formation (see intermediate **III**) and

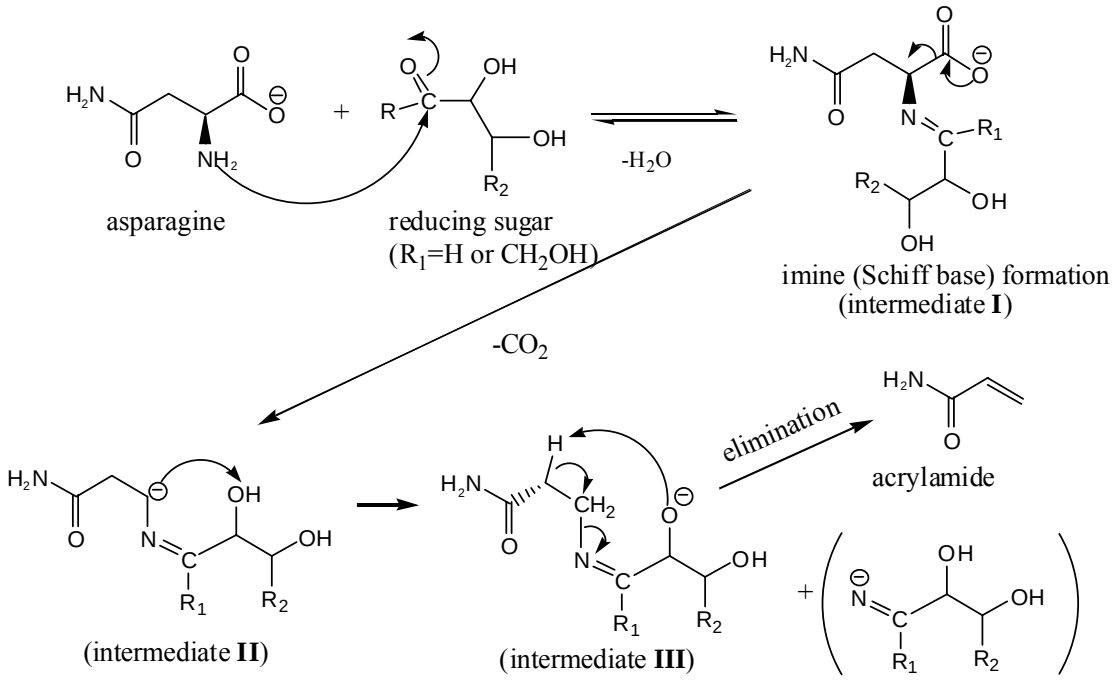

Figure 8.1. A proposed mechanism which follows the Maillard reaction, for formation of acrylamide from asparagine and reducing sugar.

thereby initiate the last step in the degradation to acrylamide.

Furthermore, in **Paper II** it was shown in the experiments were natural constituents were added to homogenized potato, and then heated, that fructose increased the acrylamide content about twice as much as other reducing sugars tested (glucose and glucose-6-phosphate). This result might be explained by the fact that fructose has two α-hydroxylic groups, the other sugars have one, and that the accessibility to the α-hydroxy group is crucial for the reaction mechanism proposed in Figure 8.1. However, this result could also be explained by other mechanisms, i.e., that fructose has a higher reactivity than e.g., glucose, a fact that is well known in the Maillard reaction.

The interest of how asparagine reacts on a molecular basis have also increased, since the asparagine together with a few other amino acids are capable of reacting with mono- and disaccharides, to form N-glycosidic bonds in addition to the formation capacities of acrylamide, where formation of N-glycosidic bond is a prerequisite (Rassolian, *et al*., 2003).

When acrylamide is analyzed in food, it means that it is the net formation of acrylamide that is analyzed, i.e. the result of formation and disappearance. It has been shown that prolonged heating time and higher temperature decreases the net formation of acrylamide (**Paper II**; Biedermann, *et al*., 2002a, 2002c; Weisshar, 2004). It is still unclear if this is the same type of reaction, which makes acrylamide unstable during prolonged storage of food (Hoenicke & Gatermann, 2004, 2005; Delatour, *et al*., 2004; Eriksson & Karlsson, 2005). It has also been shown that in model system, the acrylamide formation decreases in prolonged temperature treatment (Ehling & Shibamoto, 2005).

8.2 Alternative routes for formation of acrylamide

Even though formation of acrylamide in foods has its dominating route through asparagine and reducing sugars, there are also other minor suggested routes for the formation, routes via below components that react with formed available amino-groups from the Maillard reaction system, Figure 8.2.

Analytical work has to be performed to find if the chemicals involved in the alternative formation routes of acrylamide are common in any food items.

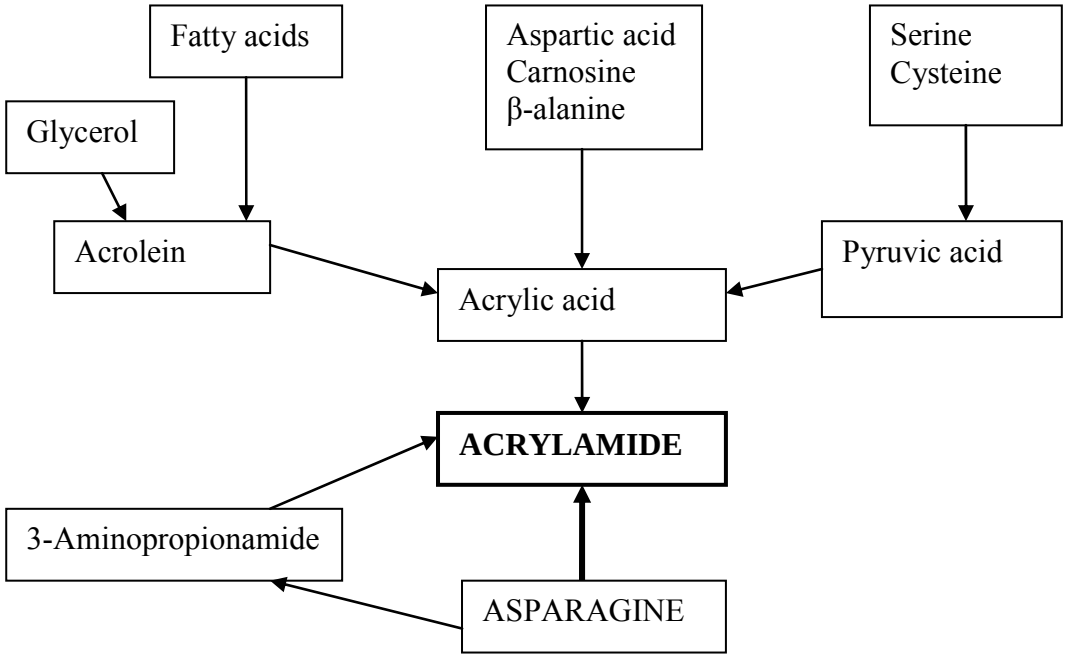

Figure 8.2 Formation routes of acrylamide.

8.2.1 Acrolein

Acrolein (1-propenal) is a simple α,β-unsaturated aldehyde, which is used for many purposes. This includes as a biocide for aquatic weed control and usage as an intermediate in the synthesis of many organic chemicals. Thus, it is

often present in commercial products as a trace impurity. It has been found in several types of foods. Acrolein is proposed to get form from dehydration of glycerol when animal and vegetable fats are heated (Umano & Shibamoto, 1987). Acrolein is found to be produced by polyunsaturated fatty acids during enzymatic and nonenzymatic maturation caused by lipid oxidation processes. Acrolein is supposed to be a probable cytotoxic compound, and analytical methods for acrolein in food are available (Casella & Contursi, 2004). In oil and fat, an alternative pathway for formation of acrylamide through acrolein has been proposed (Yasuhara, *et al*., 2003**)**.

8.2.2 Acrylic acid

Aspartic acid, carnosine and β-alanine can give rise to acrylamide through the formation of acrylic acid during their thermal decomposition (Stadler, *et al*., 2003; Yaylayan, *et al*., 2004; Yaylayan *et al*., 2005) in combination with available ammonia, to convert acrylic acid to acrylamide. The most efficient ammonia generating amino acids under thermal treatment nutrients are asparagine, glutamine, cysteine and aspartic acid (Sohn & Ho, 1995). There are to my knowledge still no reports of acrylic acid contents in food products.

8.2.3 3-Aminopropionamide

3-Aminopropionamide was first identified as a transient intermediate during acrylamide formation from asparagine (Zysak *et al*., 2003). In addition to that 3-aminopropionamide can be formed in foods by enzymatic decarboxylation of asparagine (Granvogl *et al*., 2004; Granvogl, Köhler & Schieberle, 2005), and in reactions between asparagine and pyruvic acid (Stadler, *et al*., 2004), and is a very effective precursor of acrylamide formation under certain reaction conditions.

8.2.4 Pyruvic acid

Dehydration of serine alone or in presence of sugars can generate pyruvic acid (Wnorowski & Yaylayan, 2003). Cysteine can also generate pyruvic acid, when it has lost hydrogen sulphide. It can then be a proposed reduction of pyruvic acid into lactic acid, and further dehydration into acrylic acid. Model studies with lactic acid have indicated that such transformations are possible in the presence of ammonia; mixtures of lactic acid and ammonia salts produced lactamide, acrylic acid and acrylamide when pyrolyzed (Yaylayan *et al.,* 2005).

8.3 New compounds

As a consequence of the work with acrylamide, an active search for other similar compounds has started. Until now, two components have been discussed.

N-methylacrylamide was discovered as a new suspected toxicant in food products during mechanistic trials with acrylamide in cooked meat (Yaylayan, *et al*., 2004). Its toxicological significance is still not known. The amino acid creatine is a good source as a methylamine donator, in the acrylic acid pathway for acrylamide a formation, giving rise to N-methylacrylamide (Yaylayan, *et al*., 2004).

3-Buteneamide has been discussed as a proposed similar compound, even though it has not been found yet (Biedermann, *et al*., 2003). The search has been in potato products, which has asparagine as the major free amino acid. 3-Butenamide can get formed in a similar way as acrylamide, but from glutamine instead of asparagine. In products, which has glutamine as the dominant free amino acid, like kale, spinach, broccoli and cauliflower, (Eppendorfer & Wille, 1996; Gomes & Rosa, 2000), the probability of its finding would be higher in heated products.

Further analytical work has to be performed, to analyze if these compounds occur in food products.

8.4 The Maillard reaction

The term Maillard reaction, or nonenzymatic browning, was named after its inventor, Louis-Camille Maillard. It is a collective term to the reaction between amines and carbonyl compounds, especially reducing sugars, Figure 8.3. It has been shown to occur in heated, dried or stored foods and in organisms, including the mammalians. In the foodstuffs, the Maillard reaction may influence many food quality parameters. It is responsible for changes in the flavour and colour formation; it may influence the nutritive value; it may result in the formation of mutagenic compounds; it may also form antioxidative products. Another outcome of Maillard reactions, also occurring *in vivo*, is ageing and complications of diabetes in the myocardium and arterial wall (has been implicated in age-related increase in cardiovascular stiffness) and ascorbic acid-induced cross-linking of lens proteins in the eyes (Ledl & Schleicher, 1990; Aronson, 2004; Ortwerth & Olesen, 1988).

8.4.1 Chemistry studies of the Maillard reaction

Mostly the Maillard reaction systems have focused on flavour generation. Particular attention has been paid to the identification of the chemical classes formed at defined stages in the systems. The kinetics of the reactions has

received much less attention. Knowledge of the chemistry of colour formation, mutagenic compounds and others as well are sparse compared with flavour formation. In later years, it has also been a search for mutagenic and other toxic compounds in the Maillard reactant system.

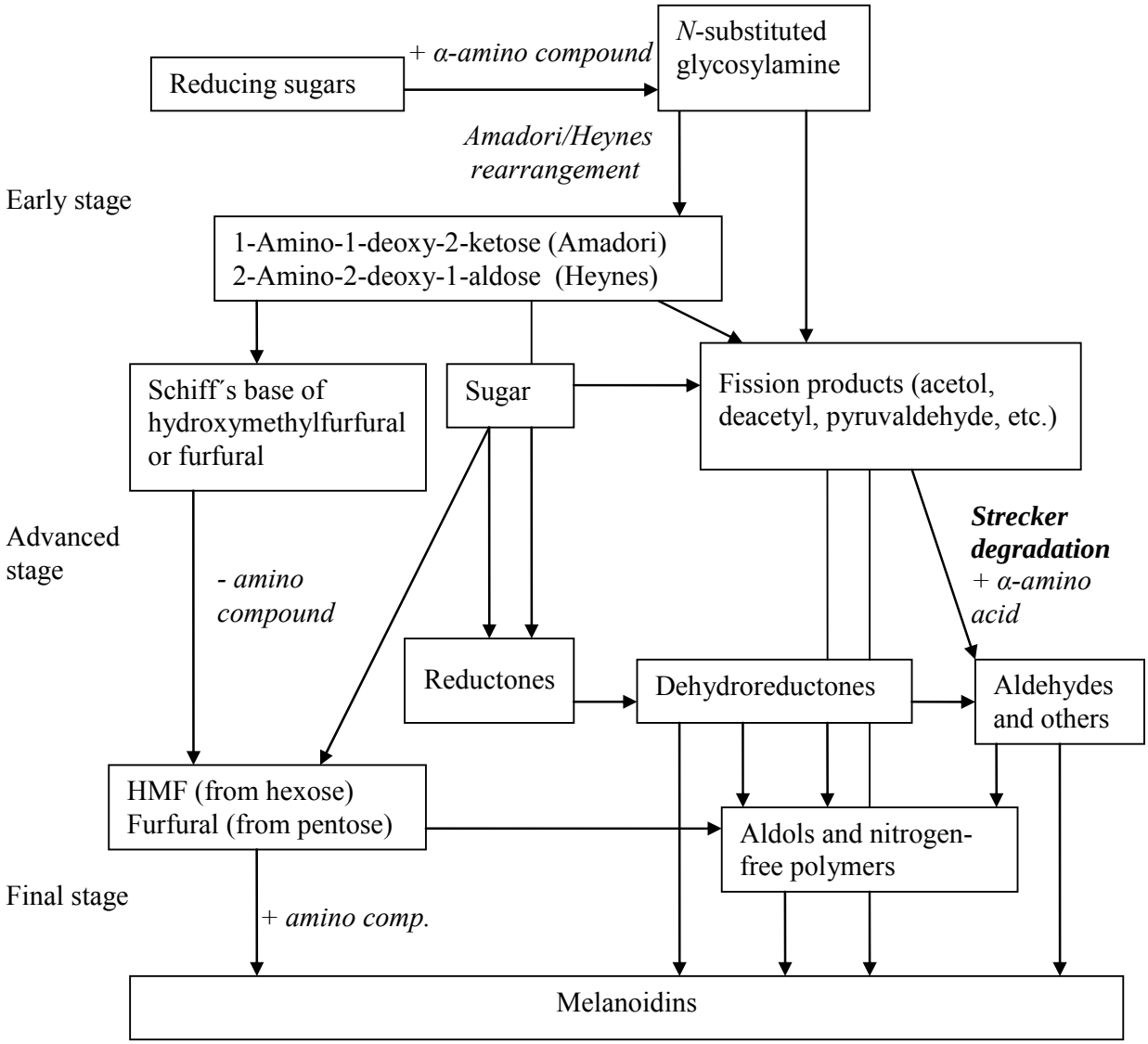

Figure 8.3 Summary of the Maillard reaction (adapted from Ames, 1990).

The Maillard reaction takes place in three major stages, and is dependent upon factors such as pH, time, temperature, concentration of reactants and reactant type.

Early stage involves the condensation of an amino acid with reducing sugars to form Amadori or Heyns rearrangements products via an N-substituted glycosylamine, Figure 8.3. It is also considered that the N-glycosylamine can

degrade to fission products via free radicals without forming the Amadori or Heyns rearrangement products. Fructosyllysine is considered as the main form of unavailable lysine during the early stage Maillard reaction, i.e. proposed as an indicator of protein nutritional damage during cooking (Ramírez-Jiménez, Guerra-Hernández & García-Villanova, 2000; Zardetto, Dalla Rosab & Di Fresco, 2003).

Advanced stage. The degradation of the Amadori and Heyns rearrangement products occurs via four possible routes involving deoxyosones, fission or Strecker degradation. A complex series of reactions including dehydration, elimination, cyclization, fission and fragmentation result in a pool of flavour intermediates and flavour compounds, Figure 8.3.

The Strecker degradation is an important pathway in which amino acids react with dicarbonyls to generate a wealth of reactive intermediates, which also is a suggested way of formation of acrylamide.

One component produced from hexose degradation is hydroxymethylfurfural. It can also be formed by dehydration fructosyllysine (Kroh, 1994; Ramírez-Jiménez, Guerra-Hernández & García-Villanova, 2000).

Final stage of the Maillard reaction is characterized by the formation of brown nitrogenous polymers and co-polymers, Figure 8.3. While the development of colour is an important feature of the reaction, relatively little is known of the chemical nature of the compounds responsible. The colour compounds can be grouped into two general classes: a) low molecular weight compounds, which comprise between two and four linked rings of heterocyclic compounds, b) the melanoidins, which have much higher molecular weights.

Colour increases with increasing temperature, with time of heating, with increasing pH and by intermediate moisture content (activity of water, a_w = 0.3-0.7). Generally, browning occurs slowly in dry systems at low temperatures and is relatively slow in high-moisture foods. Colour generation is enhanced at pH>7 (Mlotkiewicz, 1998).

8.5 Other toxic compounds formed in Maillard reactions

The Maillard reaction is responsible for the formation of mutagenic compounds formed in several degradation pathways or through Maillard reaction intermediates combining with other chemical constituents in foods. The diversity of the mutagens ranges from simple dicarbonyls compounds and heterocyclic volatiles to heterocyclic aromatic amines (Sugimora & Nagao, 1981; Wong & Shibamoto, 1996). Some of these compounds were mentioned in 1.1.4.

9 Effect of pH and enzymes on extraction of acrylamide (Paper IV, V)

The digestion of what we eat is a complex process. It includes production and use of many enzymes from our mouth, stomach and the intestine system (Brody, 1999). There is a span of around 7 pH units during food digestion, with the lowest pH in the stomach of about pH 1-2 (Fallingborg, 1999). The methodology for the analysis of acrylamide, used by most laboratories implies measurement of free water-soluble acrylamide (Castle & Eriksson, 2005; Wenzl, *et al*., 2003; Zhang, *et al*., 2005). It has so far been assumed that the water-soluble acrylamide is equal to the amount of acrylamide, that is bioavailable for humans.

We have investigated the impact of extraction methods on yield of acrylamide in food components (**Paper IV).** When enzymes (pepsin, heat stable α-amylase, protease and amyloglucosidase) have been used in the extraction procedure the obtained amounts of extractable acrylamide was similar to the yields obtained from extraction without enzymes. Others have confirmed this, that enzymatic treatment with amylase or protease did not show any effects on the results (Biedermann, Biedermann-Brem, *et al*., 2002; Jezussek & Schieberle, 2003b). However the tested enzymes is only a few compared to what we have in the gastrointestinal tract. One of the analytical methods we used for extraction was the same as for dietary fiber, which also includes incubation in boiling water. For this extraction technique there was a weak increase of the content of acrylamide, compared to water extraction at room temperature (**Paper IV)**.

In **Paper IV**, we also extracted the samples at different pH, to evaluate if pH would affect the amount of acrylamide found. In those studies, we found that when extracting at alkaline pH, the amount of measured available acrylamide monomer increased. The increase was higher for bread than for potato products (Table 9.1).

It is known since previous, that high pH affect nutrients in food, i.e. for proteins a deamidation occur, which is a hydrolytic reaction where ammonia is released. Different amino acids deamidates easier than others. One example is that glutamine deamidates at a slower rate than asparagine. Deamidation of proteins also leads to a greater solubility of many proteins, and affects their tertiary structure, which may unfold the protein, resulting in a swollen unfolded amphophilic protein molecule (Damodaran, 1996; Riha III, *et al*., 1996). Polysaccharides with carboxyl groups (pectin, alginate, carboxymethyl cellulose) are very soluble as alkali salts in the alkaline pH range. The

molecules are negatively charged due to carboxylate anions and, due to their repulsive charge forces, the molecules are relatively stretched and resist intermolecular associations (Belitz & Grotsch, 1999), while inulin is not affected by pH 12 (Kim, *et al*., 2001).

In our experiments a higher amount of acrylamide was found in food samples by changing pH towards alkaline pH, during the extraction in the analysis (cf. method in Chapter 6). One of the reasons can be that, as in polyacrylamide, it is a sterically hinder for all of the acrylamide to get into solution, during normal water extraction. By changing the pH the structure of the matrix can be changed and facilitate free acrylamide to get into solutions. Another possibility is that chemically bound acrylamide (with nucleophile groups in protein and/carbohydrates) will become available for analysis with this extraction.

The seemingly higher content of acrylamide in food obtained after extraction at higher pH than measured by commonly used method may have an impact on the daily estimated acrylamide, its its bioavailability, and finally, the health risk assessment of dietary acrylamide from food. A comparison of results with published daily intake estimations is given in Table 9.1 (Svensson, *et al.*, 2003).

Table 9.1. Comparison of estimated acrylamide intake levels in food obtained with water extraction and pH 12 extractions, Paper IV.

	Mean, water extraction (NFA*) (µg/kg)	**Intake/day (NFA*) (µg)**	**Mean increasing factor (IV) pH 12/water extraction**	**Mean, pH 12, (IV) (µg/kg)**	**Intake/day pH 12 (µg)**
Potato crisps	1360	2.8	1.6	2180	4.5
French fries	540	4.9	1.1	590	5.4
Fried potatoproducts	310	3.3	1.1	340	3.6
Cookies/biscuits	300	1.4	1.1	330	1.5
Crisp bread	300	2.0	1.8	540	3.6
Bread	50	3.5	3.9	200	13.6
Breakfast cereals	220	0.7	3.9	860	2.7
Coffee	25	12	1.7	42	20
Dried fruit,vegetables			2.9	350	
Cocoa			3.0	500	
Meat products			3.3	90	

* Svensson, *et al.*, 2003

The results in **Paper IV**, illustrated in Table 9.1 called for a necessity of a bioavailability test with mice, to compare if it is the same or different availability of acrylamide in food products analysed with water extraction and alkaline extraction (pH 12).

A background to the trials is also the knowledge about Caco-2 cells from human adenocarcinoma. They exhibit many properties of a permeable gut epithelium (Artursson, *et al*., 1996; Rubio & Seiquer, 2002). It is known from experiments with acrylamide with these cells that acrylamide monomers are highly bioavailable and passes cell monolayer via passive diffusion, and furthermore, binds to dietary proteins such as chicken egg albumin under intestinal and cooking conditions (Schabacker, *et al*., 2004). According to this it can be assumed that water extraction will be enough, especially in combination with the knowledge that the tested enzyme, **Paper IV**, treatment until now have not shown any increased acrylamide level in samples.

10 Bioavailability of acrylamide, (Paper V)

Studies of the bioavailability have to be performed so that the uptake from food can be correlated to acrylamide contents, which are relevant and reliable.

To test if the acrylamide released at alkaline extraction contributes to the amount of bioavailable acrylamide in food, mice were given different diets based on fiber-rich bread or potato (**Paper V**). The ingredients were chosen from the knowledge that extraction at pH 12 affects acrylamide content in fiber-rich bread with a factor of 3-4, and only slightly for potato.

The tested components were mixed into the standard laboratory animal feed to 15 and 45% of total weight to give two intake levels of acrylamide in each diet. Firstly, commercial potato powder for mashed potato were heat-treated, to obtain a high content of acrylamide, and secondly, commercial dark soft whole kernel bread with high content of acrylamide was chosen for the test. French loaf bread with very low content of acrylamide was used as filler together with the heat-treated potato powder in the "potato" diet. The two bread products were dried at 65 ºC, milled to a fine powder, mixed homogenously with standard laboratory animal feed and baked. The diets were analyzed with both the normal water extraction and with extraction at pH 12 (**Paper IV**) and the acrylamide intakes based on the respective analysis were calculated. The four test diets and the standard diet were given to different groups of mice (6 mice in each group). After 40 days on the diets the mice were sacrificed and the levels of Hb-adducts for acrylamide and glycidamide were measured in the blood (as a measure of the dose in blood) and compared to estimated acrylamide intake.

Acrylamide-adduct levels in mice were presented as a function of accumulated acrylamide intakes. A linear correlation (r^2=0.98) was found for the acrylamide content in the diets measured after neutral extraction. For the acrylamide content measured after alkaline extraction no correlation was found for the whole material (r^2=0.75). Instead, two separate curves, one for the fiber-rich bread and one for the potato diet, were obtained (see **Paper V**). The result shows that the acrylamide released in alkaline extraction does not seem to contribute to the dose of acrylamide in blood and the adduct level. Our interpretation is that the amount of acrylamide measured after alkaline extraction has only minor or none bioavailability.

11 Discussion of health risks of acrylamide

11.1 Toxicology

11.1.1 Exposure of acrylamide to the general population

Many countries have analysed acrylamide content in food in their markets, and there have been many intake assessments for acrylamide from food products. This has been made in several countries: Sweden (Svensson, *et al*., 2003; Norway (Dybing & Sanner, 2003), Netherlands (Konings, *et al*., 2003),), Australia (Croft, *et al*., 2004), Belgium (Matthys, *et al*., 2005), and others. Special attention has in some cases been paid to the exposure of young children and infants (Wasserbacher & Elmedfa, 2002; Tateo & Bononi, 2003; Hilbig, *et al*., 2004; Fohgelberg, *et al*., 2005). Many of these and other assessment reports, have been summarized (Boon, *et al*., 2005; Dybing, *et al*., 2005). In Table 11.1 some of the intake estimations are presented together with limits for acrylamide in different countries.

Table 11.1. Illustrates results obtained from intake estimations and as a comparison different limits for acrylamide contaminations.

Source	*Limit*	*Intake***	*Ref.*
Food, by analysis and questionnaires		Max. 0.5 µg/kg body weight /day Max. 35 µg day*	Svensson, *et al*., 2003
Food, by analysis and questionnaires		0.2-0.8 µg/kg body weight /day	Dybing, *et al*., 2005
Intake measured by Hb adducts		0.8–1.2 µg/kg body weight /day	Bergmark, 1997 Törnqvist, *et al.,* 1998 Hagmar, *et al*., 2005
Packaging material, in foods or similants	10 µg/kg		EU Commission, 2002b
Water	0.1 µg/L		EU Commission 1998
Cosmetics, body-care leave-on products	0.1 mg/kg		EU Commission 2002a
Cosmetics, other products	0.5 mg/kg		EU Commission 2002a
Air (occupational exposure)	30 µg/m^3 as OEL		AFS 2005:17

* Assuming a body weight of 70 kg; ** Non-smokers

In our work we demonstrated that acrylamide when heating biological materials, evaporates and that a fraction of formed acrylamide could be found in air both in laboratory and kitchen environments, were up to about 4 μg/m^3 acrylamide could be measured above the fry pan during frying of potato. **Paper III**, Table 1 & 3. This shows that there can be a need for air analysis of acrylamide in food production plants, like bakeries, potato chips factories, restaurant kitchens and other places where biological materials are heated, which contains components that could be precursors of acrylamide. This can be a source of acrylamide exposure, which has not been included in risk assessments reports so far (European Union Risk Assessment Report, 2002).

The Swedish occupational exposure limit (OEL) for acrylamide is 30 μg/m^3 (time – 8 hours weighed average, TWA) (AFS 2005:17). Our results from acrylamide measurements in out-stream from ovens during drying of biological materials were well below this level, although the highest acrylamide concentrations in the air from the ovens were in the same magnitude that has been seen for laboratory personal working with acrylamide and polyacrylamide gels (Pantusa *et al*., 2002). These results show that localities where dry matter determinations are made have to be well ventilated.

Carry-over of acrylamide from mother to child (via breast milk) has been reported in two studies. One study demonstrated a carry-over from food matrix into human milk by giving nursing mothers potato crisps and afterwards measure acrylamide concentrations in milk (Sörgel, *et al*., 2002). Another study showed that acrylamide passes over from mother to child. By measuring the specific acrylamide Hb adduct in the blood of mothers and in the corresponding umbilical cord blood of neonates it was shown that acrylamide passed over to the neonates. There were higher concentrations in the cord of smoking mothers than of non-smokers (Schettgen, *et al*., 2004).

For cows, the transfer from feed to milk of acrylamide was about 0.24%, with a mean half time of 2.8 hour (Pabst, *et al*., 2005). Though when acrylamide caused maternal toxicity in rats, no offspring effects during lactation were seen except from inanition due the maternal toxicity (Friedman, *et al*., 1999). In Japanese quails (*Coturnix coturnix japonica*), a carry-over from feed to egg feed-borne acrylamide was found (Kienzle, *et al*., 2005).

11.1.2 Observationsof acrylamide exposure in humans

Workers exposed to considerably higher acrylamide concentrations have in several studies shown symptoms from the peripheral nerve system (PNS) (Bachmann, Myers & Bezuidenhout, 1992; Deng, *et al*., 1993; Calleman, *et*

al., 1994). There are also examples, that persons working with acrylamide, including laboratory staff, have developed contact dermatitis (Bang Pedersen, Chevallier, & Senning, 1982; Lambert, Matthieu, & Dockx, 1988; Dooms-Goossens, Garmyn, & Degreef, 1991; Beyer, & Belsito, 2000; Aalto-Korte, *et al*., 2002.

In epidemiological studies for different types of cancer, it has not been possible to find positive evidence for relationships between dietary acrylamide and cancer. Neither in hospital-based case-control studies (Peluchhi, *et al*., 2003; Pelucchi, *et al*., 2005), nor in epidemiological studies for large bowel, kidney and bladder (Mucci, e*t al*., 2003a), renal cell cancer (Mucci, *et al*., 2004) and colorectal cancer in women were observed (Mucci, Adami & Wolk, 2005). However, the statistical power of standard epidemiological studies is expected to be far too low to detect an increased risk for cancer due to background exposure to acrylamide (Hagmar & Törnqvist, 2003, Hagmar *et al.,* 2005; cf. also Mucci, *et al*., 2003b). However, these epidemiological studies have obtained high medial interest. Recently it was reported that events before puberty may affect adult risk of breast cancer and that an increased risk of breast cancer was observed among woman who had frequently consumed French fries at preschool age (Michels, *et al*., 2005). Further studies are needed to evaluate these findings.

11.1.3 Risk assessment of acrylamide

The NTP-CERHR expert panel report concluded that it is a negligible concern for adverse reproductive and developmental effects from exposure to acrylamide in the general population (Manson, *et al*., 2005). In a Californian summary, no significant risk level, NSRL, was by regulation defined as the daily intake level posing a 10^{-5} lifetime risk for cancer and it was believed that cancer is the most sensitive health endpoint for acrylamide. NSRL was estimated to be 1.0 μg/day for acrylamide (OEHHA, 2005).

One easy way to express toxicological risk is to use MOE, Margin of Exposure, which is a term to compare body burden levels to levels where adverse effects are estimated to occur. This is calculated by dividing the highest tested dose in animals that does not cause adverse effects (NOEL), by the estimated human exposure, or in similar ways (Doull & Rozman, 2000). The benchmark MOE would be 100 to assure that there is reasonable certainty that the effect will not occur in exposed people. Chemical risk factors identified in foods can have MOE from <100 to 1000000 (Hamscher, 2004). For acrylamide MOE is estimated to be between 75 and 300, depending on average or high intake of acrylamide through food (JECFA, 2005).

After assembling available information about toxicological assessment of acrylamide in food, the Joint FAO/WHO expert committee on Food Additives (JECFA) and the International Food Safety Authorities Network (INFOSAN), 2005 summaries, which also has been agreed upon by EFSA, 19 April 2005:

- JECFA has determined that the estimated intake of acrylamide from certain foods may be of human health concern.
- Consumers, who eat large amounts of certain fried, roasted and baked foods may have an increased risk of cancer.
- Efforts to reduce acrylamide levels in foodstuffs should continue, and, specifically, the food industry and other researchers should be encouraged to share information about new technologies that can achieve this goal.
- Consumers should eat a balanced and varied diet, which includes plenty of fruit and vegetables.

A lot of actions have been taken by food industries (CIAA, 2004), universities and individual researchers, to decrease the exposure of acrylamide from food intake, and to find ways to reduce the formation of acrylamide in food products. About 50 different internationally patents have been registered, how to decrease acrylamide amount in food products (www.heatox.org). This also includes parameters reducing the formed amount of acrylamide.

12 Conclusions

The objecives of my thesis was to verify the indicated occurrence of acrylamide formation in heating of food, to identify factors affecting the formation, and to identify important sources of acrylamide exposure from food.

GC-MS and LC-MS/MS methods were developed for the analysis of acrylamide in food. The developed methods showed a high correlation coefficient (0.99), sufficient sensitivity and reproducibility. Chemically independent methods for the confirmation of the results, was a prerequisite for this work, in which an industrial chemical was found in high concentrations in food.

We demonstrated that acrylamide occurs in heated food products, with unexpectedly high levels in certain food products (up to mg/kg level in potato products). The identity of acrylamide was confirmed by our developed methods.

With potato as a food model, different factors affecting the acrylamide formation were tested. It was shown that the addition of asparagine and fructose, as well as heating temperature and time, had a large impact on the formation. Other factors affecting the acrylamide content were pH, addition of other amino acids apart from asparagine, protein and other reducing sugars. No significant effects were observed from addition of neither antioxidant nor radical initiators.

We discovered that acrylamide could be formed during heating of biological materials similar to food, also at temperatures below 100 ˚C. Furthermore, it was demonstrated that a fraction of acrylamide evaporates during heating, similar to conditions for cooking in household kitchens, and during dry matter determinations in laboratories (65-130 ˚C).

The method for extraction of food was studied with regard to occurrence of acrylamide. We showed that extraction at pH ≥12 gives higher amount (3 - 4 times) of acrylamide in fibre-rich foods compared to normal water extraction. Extraction at acidic pH or with enzymatic treatment was also tested, showing no significant effect on available concentration acrylamide.

In a study with mice, we compared the bioavailability of acrylamide extracted with the normal water extraction and at alkaline pH. It was shown that the extra acrylamide released at alkaline pH gave insignificant contributions to the *in vivo* dose, measured by hemoglobin adducts.

Our work, particularly Paper I, but also Paper II, have had a large impact on the knowledge about acrylamide, formation, occurrence, and have been discussed by the researchers in the field. Paper III – IV contains information about the behaviour of acrylamide, which mostly has not been discussed so far.

The results from Paper III shows that there is a need for investigations of acrylamide in air at food production plants, like bakeries, potato chips factories, restaurant kitchen and other places were larger amounts of materials, which contains components which are known to be able to give rise to acrylamide, are heated. This may be a non-negligible source of acrylamide exposure, which has not been considered in risk assessment of acrylamide until now. During the conditions of exposure through air reported in this thesis the potential amount of inhaled acrylamide is much lower compared to intake via food.

This may be the first example when unexpectedly an industrial toxic chemical appears to be common in food, but maybe not the last!? I am quite sure that the outcome would have been completely different if the high acrylamide levels had been found in a single food product, not belonging to European staple food and main food production.

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För 50+ personer som ger sig i kast med att avsluta ett doktorandarbete, så är det en del i en livsnerv, och den livshistoria som den personen befinner sig i, på ett annat sätt än när man direkt från universitetsexamen, ger sig i kast med uppgiften.

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